Biology of Hirschsprung Disease: Pathomorphological, Histochemical, Immunohistochemical and Genetic (RET Gene) Study of the Enteric Nervous System

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Dedication…

To my parents,

For what I have become today; none of this would have been possible without their constant prayers & support

To my wife,

For the numerous sacrifices, both personal & professional, thank you!

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DECLARATION BY THE CANDIDATE

I hereby declare that the thesis titled **'Biology of Hirschsprung Disease: Pathomorphological, Histochemical, Immunohistochemical and Genetic (RET Gene) Study of the Enteric Nervous System'** is the bonafide and genuine research work which I have carried out, under the guidance of Dr Usha Kini, Professor, Department of Pathology, St John's Medical College, Bangalore.

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List of Abbreviations

ACh	Acetylcholine
AChE	Acetylcholinesterase
APES	Aminopropyltriethoic Silane
ARM	Anorectal Malformation
BLAST	Basic Local Alignment Search Tool
CCHS	Congenital Central Hypoventilation
DAB	Diaminobenzidine
dNTPs	deoxyribonucleoside triphosphates
ddNTPs	dideoxyribonucleoside triphosphates
Dom	Dominant megacolon
DS	Down Syndrome
ECE	Endothelin Converting Enzyme
EDN	Endothelin
EDNRB	Endothelin Receptor B
EDTA	Ethylenediaminetetraacetic acid
ENCC	Enteric Neural Crest Cell
ENS	Enteric Nervous System
FG	Foregut
FFPE	Formalin Fixed Paraffin Embedded
FT	Full thickness
GDNF	Glial cell-line-derived Neurotrophic Factor
GFAP	Glial fibrillary acidic protein
GOSH	Goldberg-Shprinten
GPI	Glycosyl-Phosphatidyl-Inositol
HD	Hirschsprung Disease
HG	Hindgut
H & E	Haematoxylin & Eosin
ICC	Interstitial cells of Cajal
IERB	Institutional Ethical Review Board
IHC	Immunohistochemistry
LP	Lamina Propria
LSHD	Long-segment Hirschsprung disease
MEN	Multiple Endocrine Neoplasia

MG	Midgut
MM	Muscularis Mucosa
NABL	National Accreditation Board for Testing & Calibration of Laboratories
NANC	Non-Adrenergic, Noncholinergic
NC	Neural Crest
NCBI	National Center for Biotechnology Information
NCC	Neural Crest Cells
NF	Neurofilaments
NGFR	Neural Growth Factor Receptor
NHD	Non Hirschsprung disease
NPV	Negative Predictive Value
NSE	Neuron Specific Enolase
OCT	Optimal Cutting Temperature
PCR	Polymerase Chain Reaction
PK	Protein Kinase
PGP	Protein gene product
PPV	Positive Predictive Value
RET	REarrange during Transfection
RTU	Ready to use
SDH	Succinate Dehydrogenase
SM	Seromuscular
SNP	Single Nucleotide Polymorphism
SSHD	Short-segment Hirschsprung disease
SY	Synaptophysin
TAT	Turnaround time
TCA	Total Colonic Aganglionosis
TIA	Total Intestinal Aganglionosis
TK	Tyrosine kinase
TZ	Transition Zone

List of amino acids and their abbreviations

Amino acid	Abbreviation	
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid (Aspartate)	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid (Glutamate)	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V

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INTRODUCTION

Hirschsprung disease (HD), a type of congenital intestinal obstruction is a neural crest disorder and is characterised by the absence of ganglion cells in the enteric neuronal plexuses affecting rectum and colon. The aganglionic segment includes the distal rectum and may include variable length of contiguous proximal colon. In 80% of individuals, aganglionosis is restricted to the rectosigmoid colon (short-segment Hirschsprung disease, SSHD); in 15%-20%, aganglionosis extends proximally to include proximal colon (long-segment Hirschsprung disease, LSHD); in about 5%, aganglionosis affects the entire large intestine (total colonic aganglionosis, TCA) and may extend further to involve the small bowel. Rarely, aganglionosis may extend more proximally to encompass the entire bowel (total intestinal aganglionosis).

Though Hirschsprung disease is a fascinating disease affecting children, there is still no available incidence rate among the Indian population, probably because of lack of a registry for Hirschsprung disease in India. This reflects lack of availability of standard evidence based diagnostic modalities in hospitals and laboratories. Interestingly, an increased incidence of HD is quoted among the Asians (2.8 per 10,000 live births) in comparison to the West (1 per 5,000 live births) (1). Neonatal diagnosis of HD is still difficult and many go undiagnosed in spite of advancement in medicine and many patients suffer grave complications such as enterocolitis (1).

Since HD contributes significantly to infant mortality rate, it is justifiable to undertake an in-depth study of the disease at department of pathology which is a national referral centre for the diagnosis of Hirschsprung disease (2–8). Advancing our knowledge for its early diagnosis will help contain the disease with its impact on community and society. Hence, the need of

this study was to characterize the clinicopathologic features, innovate diagnostic modalities in the context of the Indian scenario and correlate with molecular pathology of Hirschsprung disease. This kind of maiden study with a large sample size has included various rapid tissue diagnostic modalities whose outcome would be relevant to histopathology centres of developing countries like India.

With a relative risk as high as 200, HD is also an excellent model for the approach to common multifactorial diseases. The study also aims to investigate the molecular and genetic basis of the Hirschsprung disease to gain insight into its possible pathogenic mechanisms.

REVIEW OF LITERATURE

2.1 History of Hirschsprung disease

One of the most fascinating illnesses in pediatric surgery is Hirschsprung disease. Hirschsprung disease (HD) or congenital megacolon is one of the causes for chronic constipation and presents a great challenge for pediatricians, paediatric surgeons, and paediatric pathologists. A Dutch professor of anatomy, botany and surgery named Frederick Ruysch in 1691 gave the first description of the disease. However, it was first described as a clinical entity in its own right in 1887 by the Danish physician Harald Hirschsprung. He did not, however, recognise the true cause of the disease, but merely drew attention to the dilated and hypertrophied colon proximal to the aganglionosis. Thus, Dr. Harald Hirschsprung (1830-1916) is acknowledged as the writer of the first description of two children who died of intestinal obstruction called "congenital megacolon", which is now known as Hirschsprung disease (HD) (9). Between 1880 and 1885, Dr. Hirschsprung came across two infants with similar clinical presentation. The first child had bowel problems that persisted soon after birth with absence of spontaneous bowel movements. Daily enemas and laxatives were necessary. The second case had similar bowel distensions with terminal bouts of diarrhoea variably alternating with impossibility to evacuate. Despite the fact that impacted stool was present in the colon at abdominal examination, digital examination of the rectum revealed an empty vault. He was not able to account for the pathogenesis of the disease. Notwithstanding the scarcity of information, continuous therapy was applied, but, unfortunately, both children eventually died and a post-mortem examination was performed. He was the first to explain the anatomic and clinical course of the disease. At the autopsy, the rectum was narrowed, but there was a striking dilation of the bowel loops with some ulceration of the

mucosa and associated thickening of the bowel wall (10). Surprisingly, Hirschsprung himself offered no specific treatment recommendation nor did he contribute in any other way towards solving the mystery around the disease that was named after him. Dalla-Valle was the first to point out, in papers published in 1920 and 1924, that Hirschsprung disease resulted from absence of ganglion cells in the myenteric plexus of the distal colon. At the same time, Alvarez (1922) showed that the spastic segment of the supra-anal colon was the cause of Hirschsprung disease.

Fig 1: Dr Harald Hirschsprung.

This prompted rectal biopsies for diagnosis of HD (11). Eventhough Hirschsprung disease was noted to be caused by the absence of neuronal ganglia in the narrow distal part by others (1), congenital megacolon came to be known as Hirschsprung disease only in 1988. In fact, possibly no other disease entity in pediatric surgery has caused so much confusion regarding the correct interpretation of the etiology and the necessary treatment as Hirschsprung disease.

In 1948, Swenson and Bill developed a surgical procedure (12) and the survival of patients uncovered familial transmission of HD (13). In 1973, Bolande (14) proposed the

term neurocristopathy for syndromes or tumours involving neural crest (NC) cells. HD, resulting from an anomaly of the enteric nervous system (ENS) of NC origin was, thus, called as a neurocristopathy (14,15,16).

2.2 EPIDEMIOLOGY

The major epidemiologic features of HD as a sex-modified, multifactorial, congenital trait with a population incidence of 1/5,000 live births have been described in the context of genetic studies (17). These observations may be biased in favour of familial cases and not representative of all HD cases.

Clinical and pathologic improvements have made the diagnosis of HD highly specific. Consequently, a remarkable decline in the age at which HD is diagnosed has been observed. In a survey of 487 probands born prior to 1977, 79%, 13% and 8% were diagnosed at ages, 1 year, 1 to 2 years and 0.2 years, respectively; a small number of cases escaped detection until adolescence, and rare individuals, with a mild phenotype, were identified in adulthood only (1).

Three epidemiologic survey in Baltimore County, MD, during the period 1969-1977 (18), in the province of British Columbia, Canada, during the period 1964-1982 (19), and in California during the period 1983- 1997 (19), have defined the incidence of HD based on a near complete ascertainment of cases. The incidence, expressed as the number of cases per 10,000 live births, was estimated at 1.4 among ‘‘non-whites’’ and 2.3 in “whites” in Baltimore (18), and 2.3 overall in British Columbia (19). Surveys by the California Birth Defects Monitoring Program (20) have classified by ethnicity; the incidence varies significantly with Caucasians, African- Americans. Hispanics, and Asians have rate of 1.5, 2.1, 1.0 and 2.8 per 10,000 live births respectively. Consequently, the assumed incidence of 1/5000 live births may be taken as a “representative” value.

HD has long been known to exhibit a sex bias with a preponderance in males (17). The California study showed significant variation in the sex ratio as well, with male: female ratio of 3.3, 3.4, 3.0 and 4.4 among Caucasian, African-American, Hispanics, and Asians, respectively, with a high correlation between increasing sex ratio and increasing incidence. These data suggest that rates of HD are more variable across human population in males, than in females.

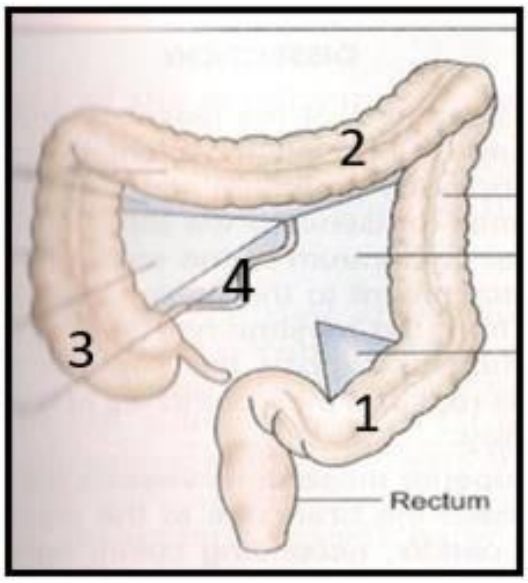
Importantly, the most significant feature correlated with segment length is the sex ratio, because the male: female rate is much higher for RSHD (4.2 to 4.4) than for LSHD (1.2 to 1.9) (1,20). The cases of these differences are unclear but are likely to include both genetic and environmental factors.

2.3 CLASSIFICATION (TABLE 1, FIG 2)

Hirschsprung disease is classified according to the length of aganglionic section (Figure 2) (Table 1). The most frequent form of HD is short segment HD (SSHD), which extends from the internal anal sphincter to variable portion of the upper two-thirds of the rectum or to the sigmoid colon. In the largest series of patients, these short segment forms represent between 75% and 80% of all HD cases. Moreover, it is the most typical form in its clinical symptoms, and that which is treated surgically by applying the most widely used techniques.

Long segment HD (LSHD) is considered to include all those cases that extend to any part of colon, proximal to the sigmoid colon, or up to the small bowel. Its various forms together account for between 20% and 25% of all HD cases. The term ‘long’ in this extent is generally used to refer to long segment HD, its most frequent from.

Total colonic aganglionosis (TCA) affects the entire colon, and usually a distal ileum segment of less than 30 cm proximal to ileocecal junction. It accounts for between 3% and 12 % of HD cases.



1. Recto- sigmoid HD (80%) / Short Segment HD
2. Long segment (20-25%) (including proximal colon)
3. Total colonic HD (5%): Colon ± SI
4. Total intestinal aganglionosis – rare.

Fig 2: Classification of Hirschsprung disease according to the length of aganglionosis (21).

The most extensively affected forms of aganglionosis involve, in addition, to the colon, major portions of the small bowel and even virtually the entire gut when it's called **Total intestinal aganglionosis.** In the longest published series, they account for 3.8% of all the cases (22–26).

Table 1. Classification of Hirschsprung disease according to the length of the aganglionic segment.

FORM	AFFECTED SEGMENT	FREQUENCY	SIMPLIFIED FORM
Ultrashort HD	<2 cm distal of the rectum	Up to 5 to 10%	75% to 80%
Rectosigmoid HD	Rectal or rectosigmoidal	>50%	
Long Segment HD	Supra-sigmoidal	>15%	LONG: 20% to 25%
Total Colonic Aganglionosis	Colon+/- 30 cm distal ileum	3% to 12%	
Total Intestinal Aganglionosis	>50 cm distal ileum	Up to 4%	

2.4 CLINICAL PRESENTATION

The diagnosis of HD is usually made in the new born period and in patients who are term-born (4 to 8% prematurity) and of normal birth weight. However, the clinical presentation of HD still depends on the patients' age. In 41% to 64% of patients, age at diagnosis is below 1 month (27). When noncomplicated, HD presents as a syndrome of neonatal intestinal obstruction with these features:

(a) abdominal distension, although it might not be an early feature (30% to 90%);

(b) vomiting (18 to 67%), with occasional bile, or blood staining

(c) failure to pass meconium within first 48 hours of life and

(d) diarrhoea and abdominal distension that is relieved by rectal stimulation or enemas. Critically, the failure to pass meconium is the first, and sometime the sole, but near constant symptom leading to diagnosis of HD in more than 60% of patients. However, this symptom can be absent or missed, so that some patients are diagnosed later in infancy and, sometimes in adulthood with severe constipation.

A specific symptom, useful in HD clinical diagnosis, is the vacuity of the rectal ampulla with explosive passage of flatus and meconium or faeces when a rectal examination is performed. Children with TCA may not present a full-blown acute intestinal obstruction syndrome and may have poor abdominal distension. The diagnosis of HD should also be considered in any infant with unexplained perforation of the caecum or appendix, a rare complication (<5%), and in any neonate presenting with enterocolitis and sepsis. Beyond the neonatal period, the prominent clinical features at presentation include constipation (68%), chronic although variable abdominal distension (64%), vomiting (37%), and a history of delayed passage of meconium (40%). Constipation is the most common presenting symptoms in older children and adults; usually, patients with late diagnosis of HD have had previous evidence of gastrointestinal dysfunction, malnutrition, or failure to thrive (28–32).

2.5 DIAGNOSTIC INVESTIGATIONS FOR HIRSCHSPRUNG DISEASE

The investigative procedures are invasive and non-invasive; the former comprising of rectal suction biopsies, while the latter include contrast enema and anorectal manometry.

Contrast enema shows the transition zone (the area between the dilated bowel and the narrow aganglionic part) in approximately 74% of cases (33). Anorectal manometry is a reliable non-invasive test (provided if it's done in standard centres) showing absence of the rectoanal inhibitory reflex in response to rectal distension in the case of HD (34).

2.5.1 NON-INVASIVE TECHNIQUES

2.5.1.1 Radiology

Plain Films

Supine, erect and lateral decubitus plain films are performed routinely depending on the clinical condition of the child; the erect film shows gaseous distension of bowel

loops and provide clues to the level of obstruction (Fig. 3). The horizontal beam film may show multiple distended bowel loops. Either can exclude perforation, if any.

The lateral decubitus view show a cone-shaped or funnel-like appearance of the transitional zone between the distended proximal bowel and the narrowed aganglionic distal segment. Contrast enema is said to be diagnostic in 80-90 percent of new borns with HD. (33,35)

2.5.1.2 Contrast Enema

A contrast enema shows a narrow rectum with uncoordinated contractions, a transition zone where the narrow aganglionic bowel joins the dilated ganglionic bowel. A delayed evacuation on a plain x-ray taken 24 hours later (Fig. 4) may show the typical transition zone. Barium enema is diagnostic in 80-90 percent of new born with HD (33,35). Interestingly, there may be no transition zone in TCA, while the colon looks small and appears foreshortened – the 'question mark' colon (36,37).

2.5.1.3 Anorectal Manometry

Introduced by Schuster 1965, (38) this method is based on the fact that in the normal child, the intraluminal pressure of the anal canal falls when the rectum is distended artificially. This phenomenon is thought to be due to the relaxation of the internal anal sphincter muscles and is called rectosphincteric reflex. In Hirschsprung disease, however, the pressure in the anus rises when the rectum is distended.

Fig. 3 A 24 hours delayed abdominal X-ray showing significant retention of contrast in the colo-rectum suggestive of aganglionosis.

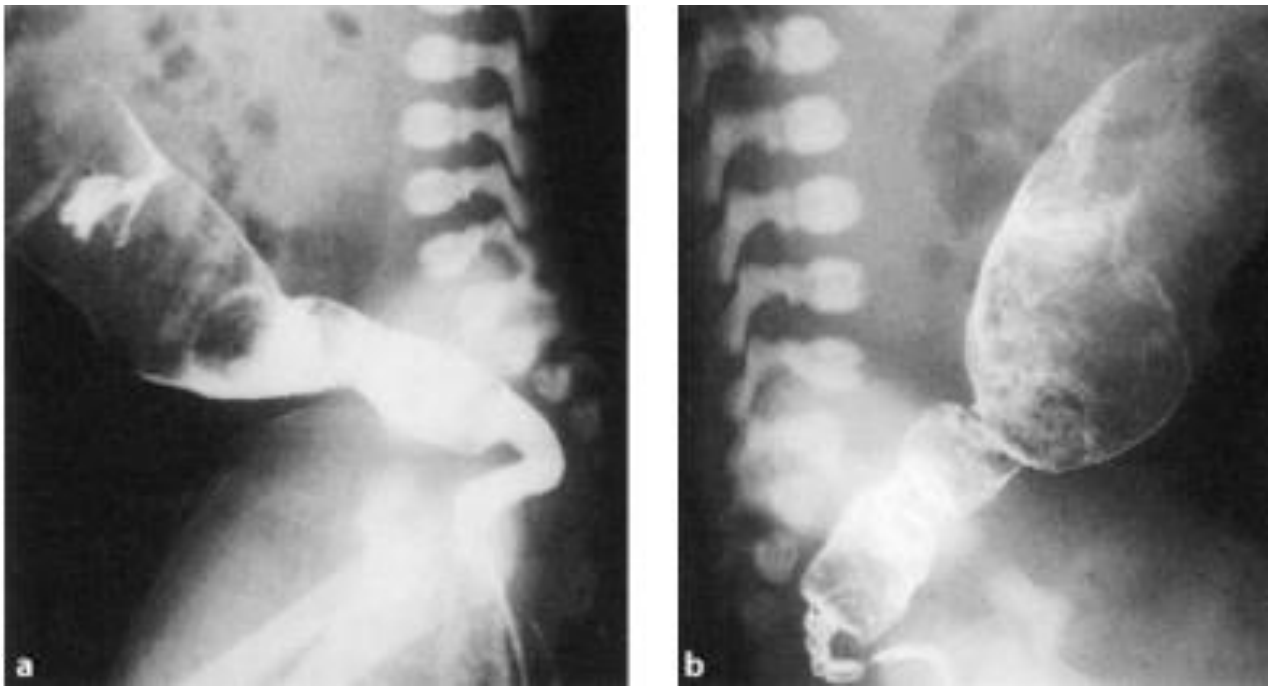


Fig. 4 a. Contrast enema shows narrow rectum with sigmoid colon gradually widening proximally to give the "tunnel funnel" appearance of Cremin. **b** Routine film at 24 hours showing significant retention of contrast in the bowel (40).

The accuracy of the method increases greatly with age of the child, as has been demonstrated by Meunier 1978 (39) in his three-year study period when he performed manometric examination on 229 children.

2.5.2 INVASIVE (RECTAL BIOPSY) TECHNIQUES

2.5.2.1 Anatomy and Histology of the Rectum (Figure 5)

The rectal biopsies are best studied and understood after a brief recollection of the histology which is as follows:

Histologically (41), the colonic consists of four layers;

1) the mucosa nearest to the intestinal lumen, which is subdivided into epithelium, lamina propria, and the muscularis mucosa,

2) the submucosa,

3) the muscularis comprising of the circular and the longitudinal muscle layers and

4) the serosa, the outer most layer.

The ENS, the second brain, made up of numerous interconnected neural networks (plexuses) of neurons and glial cells in the wall of large intestine has two components: the myenteric plexus (Auerbach´s plexus) which lies between the inner circular and outer longitudinal muscle layers which mainly innervates the intestine, and the submucosal plexus (Meissner´s plexus) which is found in the submucosa innervates the mucosa, the submucosa and, to some extent, also the circular smooth muscle fibres (42).

Interstitial cells of Cajal (ICC) found adjacent to the muscularis plexus in the intestine, control gastrointestinal motility by generating slow waves in the wall of intestine and hence, called the pacemaker cells of the intestine (43,44).

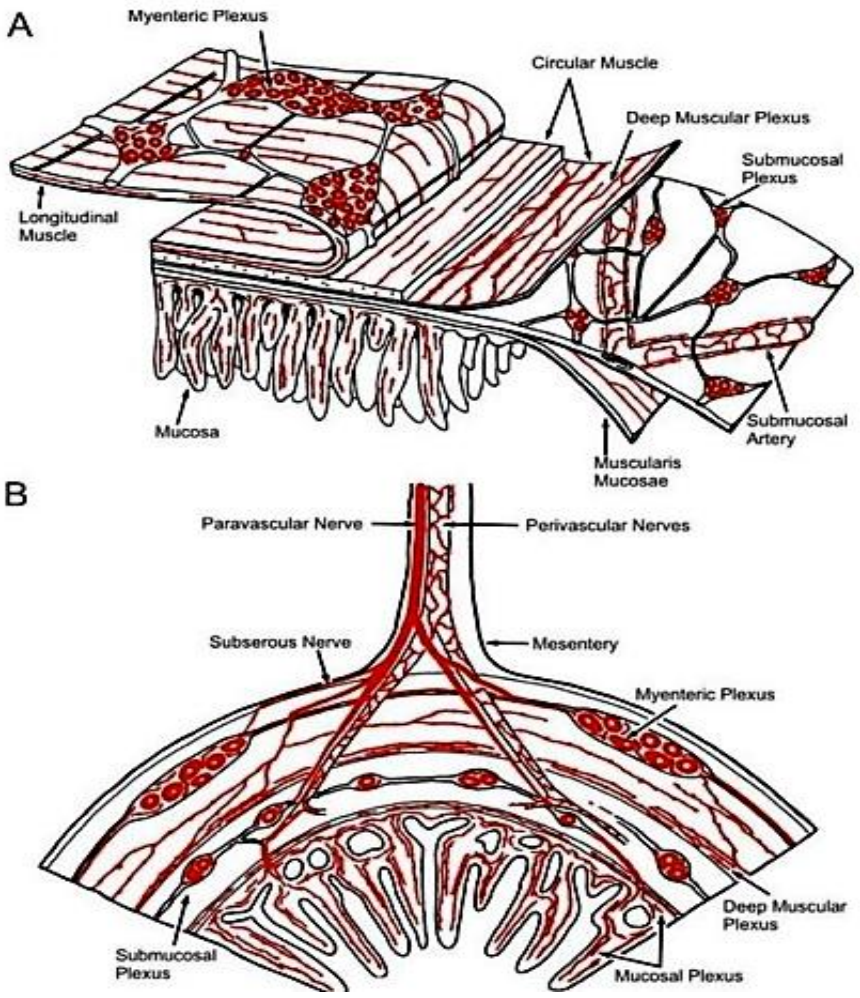


Fig 5. Histology of the large intestine showing various layers of the intestinal wall (42).

The ENS consists of three functional categories of neurons; sensory, inter- and secreto-motor neurons, required to manifest local reflex behaviour and is central to the regulation of both motor and secretory activities and independent of the CNS (45). The complexity of the ENS is further illustrated by its association with a large number of neurotransmitters. The neurotransmitters in motor neurons can be excitatory such as acetylcholine (ACh) and the tachykinins, or inhibitory as the NO, VIP, PACAP and adenosine triphosphate (ATP) (Furness 2006). VIP and ACh are also the primary transmitters of secretomotor neurons that control fluid secretion in the small and large intestines (42).

2.5.2.2 Types of Biopsies:

Rectal biopsy has a diagnostic accuracy of at least 90 per cent. For this reason, most surgeons rely on diagnostic rectal biopsy before proceeding with definitive surgical treatment.

2.5.2.2.1 Full thickness rectal biopsy:

Full thickness rectal biopsy is the most common procedure used to diagnose HD. It is based on the observation that ganglion cells in neural plexuses are invariably absent from the distal rectum of a patient with HD.

Full thickness rectal biopsy includes mucosa, submucosa and muscle coat. These are relatively large size which permit easier orientation of the specimen and make available a large number of both myenteric and submucosal nerve plexuses for evaluation. The surgeon ensures that the site of the biopsy is at least 2 cm above the dentate line to avoid sampling the normal hypoganglionic zone. Fewer sections can thus be cut from the large biopsy for assessment of nerve plexuses. This biopsy is probably most commonly indicated in children who have had more than one rectal biopsy for diagnosis. The disadvantages are that it requires general anaesthesia, may be associated with post-operative complications such as haemorrhage and perforation and lastly may result in adhesions which may interfere pull through planned at a later date.

2.5.2.2.2 Mucosal rectal biopsy:

The advantage of the mucosal biopsy is that it can be obtained in an outpatient clinic with short or no general anesthesia and is easily performed even in very small infants. This procedure permits multiple sampling and has minimal complications. It is usually done with a punch biopsy forceps or better a specially designed suction apparatus and uses a tie for guidance. The disadvantage of the

mucosal biopsy technique is that it yields only mucosal and submucosal tissue, producing a disc-shaped specimen 3 to 4 mm in diameter and 1 to 2 mm in depth. Therefore, it only contains Meissner's submucosal plexus. Auerbach's myenteric plexus, which is located between the circular and longitudinal muscular coats, cannot be evaluated.

As these biopsies are small, orientation is difficult but when done so permits visualization of submucosal ganglia which are few and far apart. Occasionally, some lymphoid follicles may occupy much of the submucosa further reducing the area of submucosa for the study. Since, these biopsies are small and submucosal ganglia are few and far apart, one needs to study 60-80 serial sections to demonstrate a ganglion cells before the diagnosis of HD given based on absence of ganglion cells. It is in these mucosal biopsies that AChE staining is particularly valuable thus avoiding serial sections study. The mucosal rectal biopsies mandate an AChE staining after the routine H&E stain.

Thus, these mucosal biopsies mandate frozen sections with required expertise and skill to cut these minute biopsies followed by AChE staining. Above all, one needs to have the confidence to distinguish diagnostic from equivocal finding and communicate the same to the clinician to plan further management (46).

2.5.2.2.3 Low level biopsies:

To exclude HD, it is required to biopsy 2-3 cm proximal to the dentate line (transition between rectal and squamous mucosa) (Fig. 6 and 7) because a number of studies have demonstrated that the distal 1-2 cm of rectum is physiologically hypoganglionic, and a justifiable concern exists that sampling of this area may lead to a false impression of aganglionosis (47–49). In practice, although the distal rectum is hypoganglionic, ganglion cells can often be found in a distal biopsy

from a patient who does not have HD if the biopsy size is adequate and sectioned thoroughly. However, this can require examination of more than a hundred histologic sections to find a single unequivocal ganglion cell. Cognizant of the physiological submucosal aganglionosis that exists in the terminal rectum, Aldridge and Campbell recommended that at least two biopsies should be obtained 2-3 cm proximal to the dentate line (47). This recommendation is generally considered the minimal requirement in most practices. However, no universal standard exists; some practitioners advocate more extensive sampling while a few do well with one.

When operating the suction biopsy device, the precise location of the biopsy port relative to the dentate line is difficult, and it is not uncommon to find that a biopsy was either at or below the squamocolumnar junction. Presumably, similar deviation may occur in the oral direction, in which case biopsies taken more proximally risk missing a short segment of aganglionosis. For these reasons, its advised to take biopsies from multiple levels (eg, 1, 2, and 3 cm proximal to the dentate line) to increase the likelihood that adequate tissue is obtained and reduce the likelihood that very short-segment disease will be overlooked (50). This strategy has effectively eliminated a problem with inadequate low level biopsies because, even if the “1 cm” biopsy is too low (squamous mucosa), the others are not and the likelihood that all three biopsies will have inadequate submucosa is negligible.

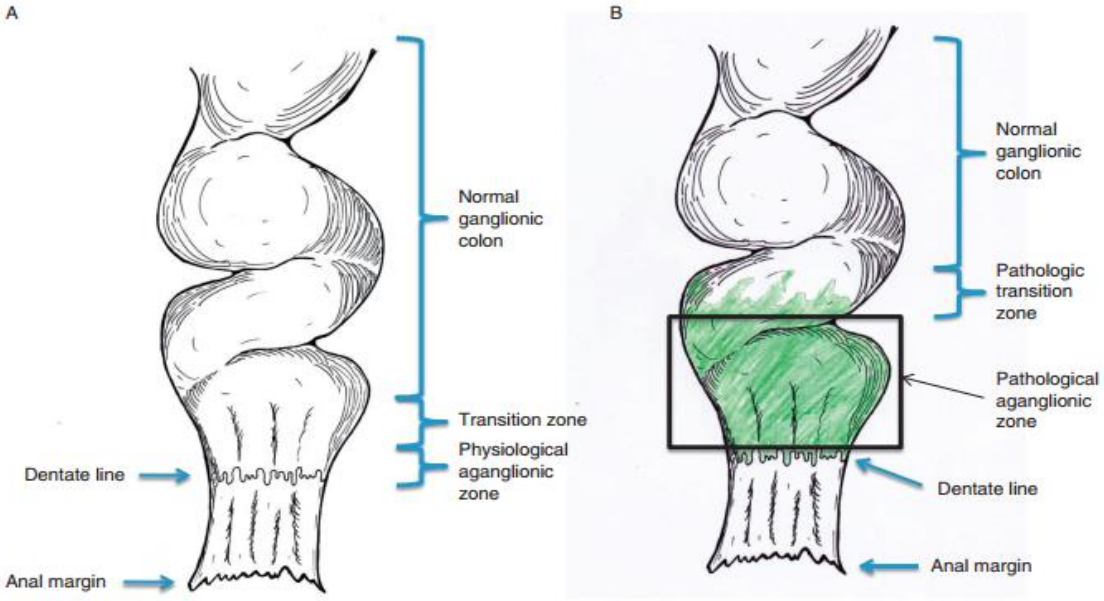


Fig 6. A, The normal rectum showing the physiological, hypoganglionic, or even aganglionic zone above the dentate line that transitions to the normal, more proximal, ganglionic bowel. Biopsies taken too close to the dentate line may be hypoganglionic or aganglionic and hence reported as false-positive for Hirschsprung disease. B, In Hirschsprung disease, the aganglionic zone extends from the dentate line proximally for a variable distance, before a transition zone leads to normal innervation (51).

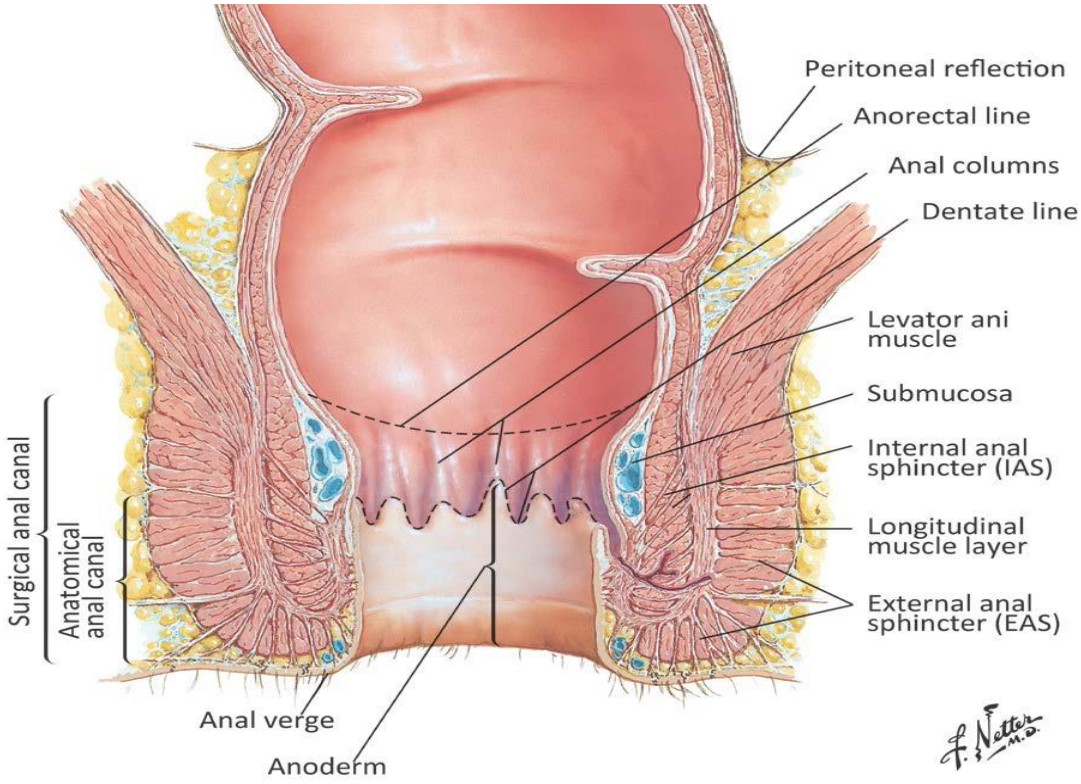


Fig 7: Anatomy of the anorectal junction (52)

2.5.2.2.4 Intraoperative seromuscular biopsies:

Many surgical approaches to HD are employed with a trend toward one-stage procedures that are often transanal. In other instances, diagnosis based on suction rectal biopsy is followed by a two-stage procedure that begins with placement of an ostomy proximal to the aganglionic segment. Intraoperative seromuscular biopsies are thus, important to determine that ganglion cells are present at the level where the ostomy or anastomosis will be placed. However, whether the definitive surgery is done in one or two stages, it is helpful if the boundary between aganglionic and ganglionic gut is localized intraoperatively before resection of any bowel. Generally, this is accomplished by sequential seromuscular biopsies, from distal to proximal.

Seromuscular biopsies should be a minimum of 1 cm in length and extend for a depth of 3-5 mm, to include the longitudinal and most of the circular layers of the muscularis propria to include myenteric plexus. Proper orientation of the biopsy for frozen sections intraoperatively greatly facilitates sampling and identification of ganglion cells. The goal is to cut the tissue perpendicular to the serosal surface, thereby visualizing both the muscle layers and myenteric plexus sandwiched between them. With a well-oriented biopsy, five serial sections are generally sufficient to confirm/ exclude aganglionosis. Recognition of ganglion cells is usually not difficult, although inflammation sometimes obscures their cytologic features.

In the operating room, the surgeon must be aware of limitations to the seromuscular biopsy. The biopsy only examines a portion of the circumference. Because the transition from ganglion cells to aganglionic bowel typically extends up to 3 cm more distally along some part of the circumference, (53,54) if ganglion

cells are seen, one should not infer from this ganglionated small seromuscular biopsy to reflect ganglionosis of the entire circumference and it may be part of the transition zone. It is assumed that 'the bowel 3 cm proximal to any ganglion cell-containing seromuscular biopsy will have ganglion cells around the entire circumference'(53). Therefore, it may be prudent to routinely resect a minimum of at least 3 cm of bowel proximal to a "positive" biopsy site for ostomy placement or pull-through (53). It is difficult, if not impossible, to distinguish a seromuscular biopsies of ganglionic bowel from that of hypoganglionic transitional zone.

2.5.2.2.5 Full circumference doughnut biopsies:

Delineation of mild-to-moderate hypoganglionosis requires near full circumference tissue samples and rigorous neuronal counts, which is not possible intraoperatively. Nonetheless, examination of a frozen section that represents the full circumference of the proximal resection margin can assess the general distribution of ganglion cells and give an insight into whether the distribution of ganglion cells is relatively uniform, and thereby reduce the likelihood of a transition zone pull-through (TZPT) (53). This is preferably done intra-operatively.

At laparotomy, multiple seromuscular biopsies can be taken from the antimesenteric taenia coli for evaluation of the innervation, beginning distal to the gross or radiological transition zone (rTZ) or from just above the peritoneal reflection and proximally till normal innervation is reported.

'Normally innervated' bowel is defined as the presence of regularly spaced, morphologically normal ganglion cells along the entire circumference of the bowel in the submucosal and myenteric plexus with no hypertrophic nerve fibers. Where normal innervation is reported on the initial representative seromuscular

biopsy for leveling; a doughnut, i.e. a circumferential full thickness ring of bowel at this level can be further evaluated (Fig. 8). Frozen H & E sections can be used for the assessment of the doughnut; and where these sections are deemed inconclusive, AChE staining can be employed. It is only after confirmation of a normal innervation along the entire circumference (all quadrants) of the bowel that the final distal level of normal bowel can be established.

If the initial doughnut is unsatisfactory, further doughnuts can be analyzed from the proximal colonic segment till normal innervation is reached. (55).

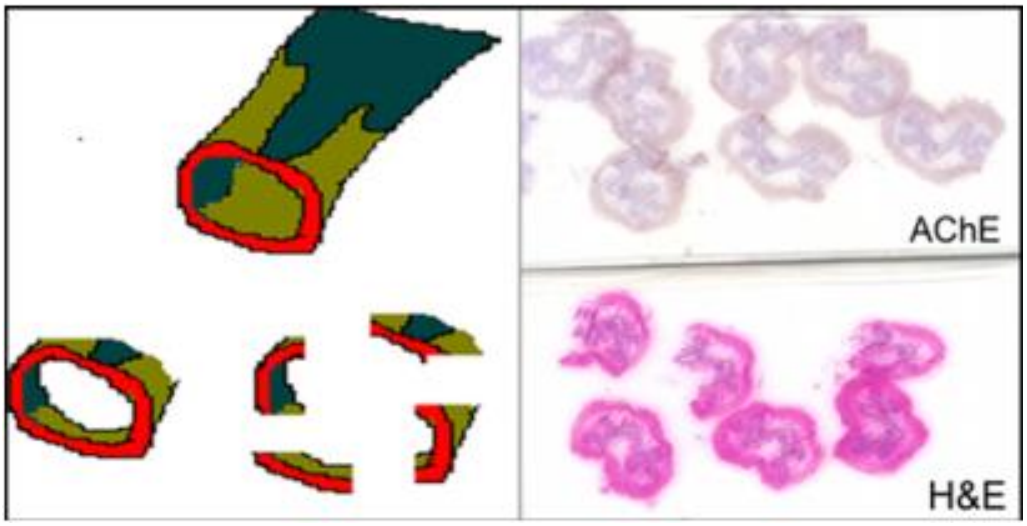


Fig 8. Schematic representation of a 'doughnut', i.e. circumferential full thickness ring of colon and a 'leading ganglionated edge' that can account for discrepancies in quadrantic innervation (left). Slide mounts of frozen section H &E, AChE staining of quadrants of a doughnut (right) (55)

2.5.2.2.6 Resected specimens:

A detailed study of resected specimen from a patient with HD aims to confirm the diagnosis of HD, measure the extent of aganglionosis, map the transitional zone and its length and assess the innervation status at the proximal end of resection. This can be done by sampling serial bits along the length of the resected segment

to document the presence/ absence of submucosal/ myenteric ganglia. One could either use Swiss roll from these longitudinal strips of the entire length or use transverse sections to assess the interface between aganglionic/transition/ ganglionic segment keeping in mind the leading edge of the neural crest migration.

2.5.3 PROCESSING OF BIOPSIES:

2.5.3.1 Frozen Section:

Frozen sections are primarily carried out for

i) Rectal mucosal biopsy followed by AChE histochemistry for primary diagnosis of HD

ii) seromuscular biopsies for leveling purposes to place a colostomy at an appropriate point where the colon is uniformly ganglionated circumferentially in the proximal surgical margin/ the proximal doughnut.

iii) mucosal rectal biopsies, leveling biopsies and the proximal margin of the resected segment in primary (endorectal) pull through/ Soave's procedure in cases of short segment HD.

2.5.3.2 Paraffin section:

i) For full thickness rectal biopsies fixed in formalin. Few complete sections are adequate to assess its proper orientation to study the neuronal plexuses.

ii) For mucosal biopsy processed previously for frozen section for further immunohistochemical workup

iii) On mucosal biopsy for careful examination of a minimum of 60-80 sections if both AChE histochemistry or calretinin immunostain are not available, for a presumptive diagnosis of HD.

2.6 SPECIALIZED DIAGNOSTIC MODALITIES FOR HD

From the surgical pathology perspective, HD is excluded if one or more ganglion cells is identified in the distal rectal biopsy. Conversely, the diagnosis of HD can be established with confidence, when hypertrophic nerves, and no ganglion cells, are identified in a full thickness biopsy. Assessment of specimen adequacy and recognition of ganglion cells and hypertrophic nerves is influenced by experience and regular reporting practice of the histopathologist. Even the most experienced pathologist will encounter some biopsies that yield equivocal results, usually because an inadequate amount of submucosa is present. Above all, a pathologist must have the confidence to distinguish diagnostic from equivocal findings and clearly communicate the results to the clinician. In some instances, rebiopsy may be necessary (53).

Two different approaches have evolved for the pathologic evaluation of rectal biopsies. The first, which is based solely on analysis of enzyme histochemistry using frozen sections to identify ganglion cells and acetylcholinesterase (AChE)-positive nerves. This strategy was pioneered by Meier-Ruge and colleagues and is employed by a relatively small number of laboratories in Europe and other parts of the world. The more widely used approach relies primarily on paraffin sections stained with hematoxylin and eosin (H&E), although many laboratories complement the latter with AChE histochemistry and/or paraffin-based immunohistochemistry (56). Although well-controlled comparative data are not available, the different approaches appear to be equally accurate and reliable. Adequacy of the biopsy and technical and interpretational experience of the laboratory are probably the most important variables to influence diagnostic accuracy.

At a minimum, a diagnostic mucosal biopsy measures 3 mm in diameter with at least one-third submucosa (Figure 9A). When properly oriented and sectioned adequately

(50-75 sections), H&E-stained, paraffin-embedded sections are generally sufficient to exclude the presence of submucosal ganglion cells to suggest a diagnosis of HD (50). The presence of thick and prominent (so called as hypertrophic measuring 40 m diameter) nerve fibers observed in many, but not in all cases with no ganglion cells, favor the diagnosis of HD (Figure 9B) (57). The hypertrophic nerves that exist in most patients with HD arise from extrinsic autonomic and sensory fibers, which enter along with vessels from the perirectal region and project for a finite distance rostrally (58). It is the number and diameter of these fibers that increase in HD, giving rise to the "hyperplastic and hypertrophic" nerves that are frequently, but not always, observed in the submucosa and myenteric plexus of HD patients. Because these fibers project only a finite distance proximal to the rectum, hypertrophic innervation may not be observed in biopsies taken rostrally in long segment disease (58). Furthermore, extrinsic nerve hypertrophy of the rectum may not be observed in patients with combined deficiency of intrinsic ganglion cells and other peripheral ganglia (more common with long-segment HD) or very premature infants with delayed extrinsic innervation (59). Sometimes, some biopsies are suboptimal due to paucity of submucosa, crush artifact, or both. In several series dating back to the original suction rectal biopsy paper by Dobbins and Bill, (60) reported rates of inadequate biopsies have generally lowered between 10% and 20% (61,62).

It is on these mucosal biopsies which need confirmation of abnormal innervation that AChE histochemistry is mandatory.

2.6.1 Acetylcholinesterase histochemistry:

Acetylcholinesterase (AChE) staining is the gold standard for a diagnosis of HD and has now become routine for all rectal biopsies in the initial assessment of suspected HD. Karnovsky and Roots reported their technique in 1964 (63) which was put to use

on rectal biopsies by Meier-Ruge in 1972 (11). Kobayashi et al proposed an accelerated modified method, using (DAB) reagent and 4- chloro-1-napthol and osmium as reagents. Further rapid AChE by Kobayashi required 6 minutes of incubation but used DAB which is a hazardous substance as a chromogen. Kobayashi et al based this technique on oxidation reduction reactions. Addition of hydrogen peroxide speed up the copper ferrocyanide catalyzed oxidation of DAB (64). Martuciello et al.(65) performed rapid AChE staining using 3-amino-9 ethylcarbazole(AEC) as chromogenic substance. It required a short incubation time of 8 minutes. This procedure avoided the use of DAB and napthol and thus was considered safe for handling.

The disparate results of the four different investigative groups show that the method of AChE is not completely uniform. It was concluded earlier that AChE reaction is definitive for the diagnosis of HD if only the results are positive. This problem to some extent was overcome by the use of rubeanic acid by Goto et al in the AChE reaction (66).

Kobayashi et al in 2007 (64) introduced a new rapid acetylcholinesterase staining kit for diagnosing HD. This allowed AChE histochemistry to be performed rapidly with complete accuracy, without any risk of toxicity. However, DAB was used in both their rapid technique published earlier and the new rapid kit.

The duration of rapid AChE stain was further modified by Kini et al (3). The duration of rapid AChE stain was reduced to 30 minutes by modifying the incubation solution of Karnovsky and Root's method and carried out on cryostat sections rather than snap frozen sections used by Goto et al (66). The addition of rubeanic acid made the staining crisp, permanent, specific with no background staining.

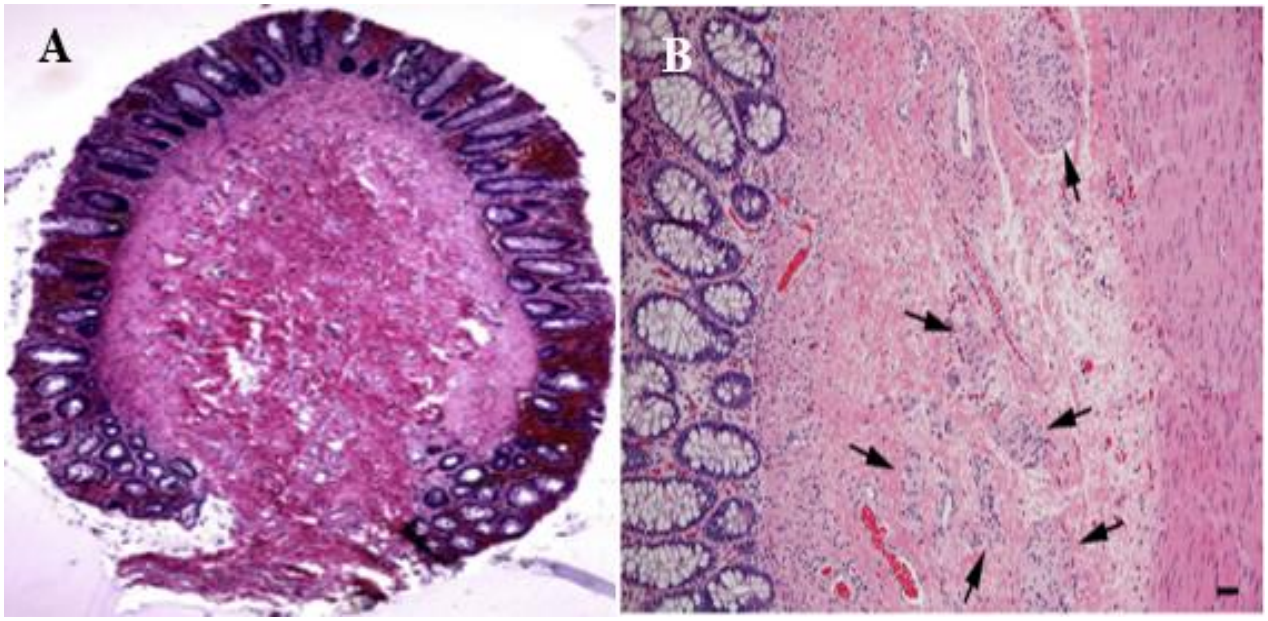


Fig. 9 Histopathology of HD. (A) An adequate suction rectal biopsy should be 3 mm in greatest dimension, and at least one-third of the biopsy should be submucosa. (B) Hypertrophic nerves (40 m) are typically present in the aganglionic submucosa in HD (67,68).

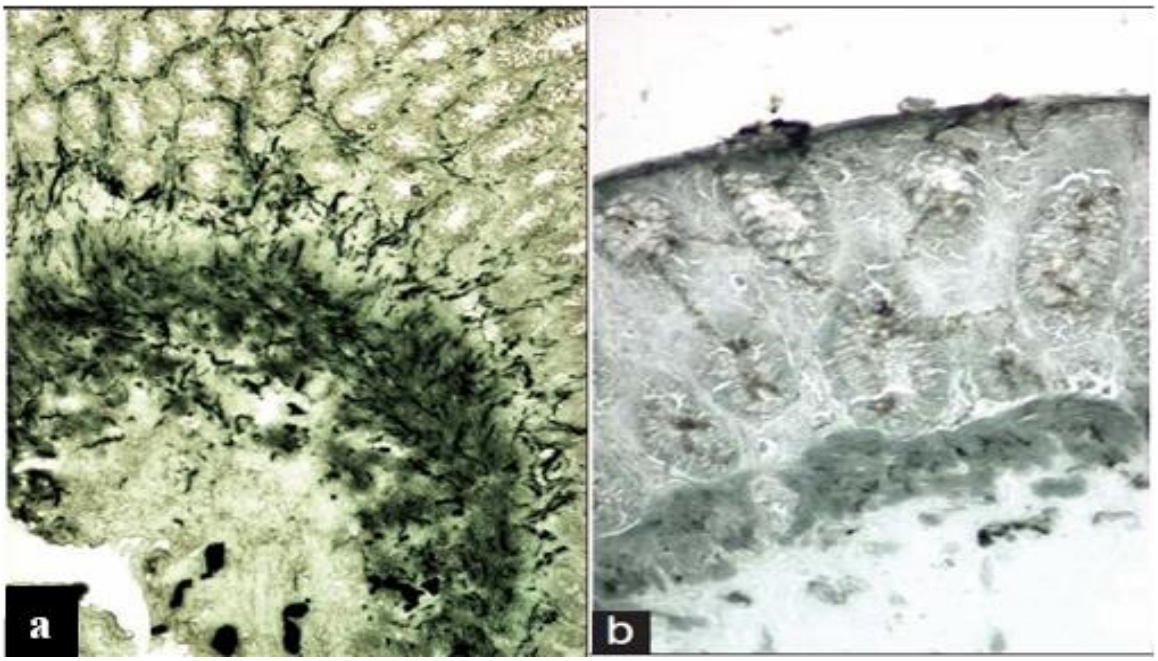


Fig. 10 a) Rectal suction biopsy from a case of Hirschsprung disease. Lamina propria and muscularis mucosae show prominent AChE activity. **b)** Rectal suction biopsy from a normal control. Within the lamina propria AChE activity is barely detectable. The muscularis mucosae show a nonspecific esterase activity (2).

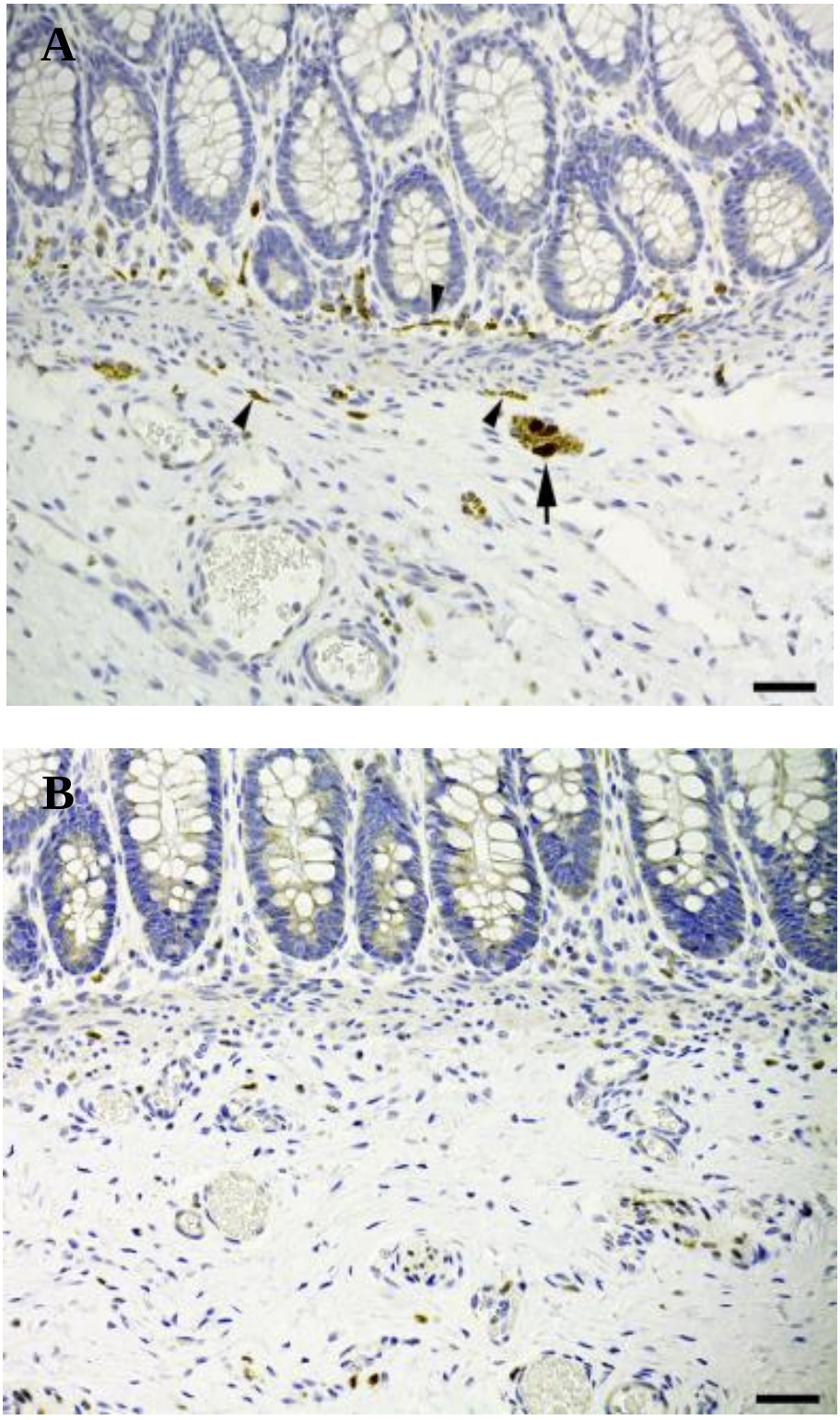


Fig 11: (A) Calretinin immunoreactivity exists in small nerves in the muscularis mucosae and lamina propria (arrowheads), when ganglion cells are present. (B) No calretinin immunoreactivity is present in the mucosa or superficial submucosa of aganglionic bowel (68).

This approach of staining is of advantage when they are confronted with inadequate biopsy material. Despite the absence of submucosa, strongly positive AChE activity within the lamina propria is diagnostic of Hirschsprung disease (11).

The use of AChE evaluation for specimens obtained from suction biopsies demonstrates that associated with the absence of submucosal ganglion cells is a substantial increase in AChE activity within the lamina propria and muscularis propria (Fig. 10 a). Thus, in the absence of submucosal ganglion cells, there exists an overabundance of acetylcholine and an excess of the corresponding enzyme AChE. Therefore, in the narrowed abnormal segment of Hirschsprung disease, an excessive accumulation of the enzyme occurs resulting from a continuous release of acetylcholine from the axons of the extramural parasympathetic ganglion (69).

Under normal conditions, barely detectable AChE activity is observed within the lamina propria, muscularis mucosae, and submucosa. Although the enzyme is present, if colonic innervation is intact, enzyme activity is so minimal that its presence is virtually imperceptible (Fig. 10 b). In the myenteric plexus, AChE activity is clear and characterizes the presence of ganglion cells and parasympathetic nerves.

The slides stained for AChE can be evaluated microscopically using the criteria suggested by Challa *et al.* and Babu et al (70,71) as follows:

Positive pattern A*:* Presence of prominent (hypertrophic) AChE positive fibres in the submucosa extending through the muscularis mucosa into the lamina propria akin to an arborizing tree trunk.

Positive pattern B*:* Presence of such nerve AChE positive fibres in the muscularis mucosa and the lowermost portion of the lamina propria below the region of the basal crypts.

Equivocal pattern*:* Presence of AChE positive nerve fibers in the submucosa alone with no specific pattern in the LP. The AChE activity may be normal or increased.

Negative pattern: Absence of stained nerve fibers in the mucosa with or without occasional obscure nerve twigs in the muscularis mucosa and submucosa.

A positive biopsy for Hirschsprung disease is one which shows increased AChE activity of Pattern A, B and Equivocal patterns.

An additional advantage in using AChE activity evaluation is the possibility of estimating the extent of the pathology. Not only may aganglionosis be firmly established, but the surgical margin of the proximal segment after resection may indicate its suitability for anastomosis and thus exclude hypoganglionosis/ transition zone (69).

The only disadvantage of this AChE staining technique is that it requires frozen sections as the enzyme is destroyed beyond 37ºC and requires technical support and expertise to handle minute biopsies and to interpret the results. In such situations, one requires an additional biopsy if paraffin sections are also going to be evaluated (72).

2.6.2 Immunohistochemistry and the diagnosis of Hirschsprung disease

A large number of papers have been published that essentially describe some neuron-specific marker that the authors suggest could be used to facilitate the diagnosis of HD (73). Although most of these antibodies are specific, none is used widely in practice of reporting Hirschsprung disease because there are no specific markers for acetylcholinesterase enzyme on formalin fixed biopsy and pathologists who regularly search for ganglion cells in H&E stained sections are quite good at discriminating neuronal cell bodies without the need for special stains for ganglion cells. False-positive diagnoses result either from inadequate sampling or observer inexperience.

Immunohistochemical markers that have been used for research and clinical diagnosis of HD are:

Calretinin:

Among the many antigens expressed by enteric neurons, the calcium-binding protein calretinin is a potentially useful immunohistochemical target for immunohistochemical studies that complement traditional H&E based diagnosis of HD (74). Calretinin immunoreactivity is normally present in small intrinsic fibres located in the muscularis mucosae and lamina propria (Figure 11 A). In the mucosa of aganglionic bowel, expression is completely absent (Figure 11 B). The results of a recent case-control study suggested that calretinin immunohistochemistry is at least as sensitive and specific as AChE histochemistry for the diagnosis/exclusion of HD from suction rectal biopsies (75). In contrast with AChE staining, which requires a second frozen biopsy and specialized methods, calretinin immunostaining can be performed on paraffin sections of formalin fixed biopsies with methods that are available in most pathology laboratories.

Neuron Specific Enolase (NSE):

Neuron-specific enolase (NSE) is exclusively localized within neurons of mammalian nervous tissue. NSE is said to be a selective marker of the degree of neuronal maturity since this molecule is expressed by neurons when they have initiated their specific metabolic and synaptic activities. NSE immunohistochemistry leads to intense staining of ganglia which allows the recognition of small ganglion cells and the overall pattern of micro innervation since it also stains nerve fibers within the circular muscle of the bowel (76–78) .

Protein Gene Product (PGP) 9.5:

The brain-specific protein PGP 9.5 is one of the most sensitive markers for identifying ganglion cells. Therefore, PGP9.5 is a reliable marker for ganglion cells and nerve fibers both of normal and hypertrophic in the mucosal and submucosal plexus in bowel biopsies (76,79) .

Cathepsin D:

Cathepsin D is a member of a family of lysosomal acidic proteinases which play a major role in the intracellular catabolism of proteins. Cathepsin D catabolizes neuropeptides such as substance P (SP), somatostatin, β-lipoprotein, and angiotensinogen. Ganglion cell bodies within the submucosal and myenteric plexus of the human intestine showed intense granular cytoplasmic immunoreactivity for cathepsin D. No cathepsin D-immunoreactive cells were detected in aganglionic bowel. Cathepsin D does not stain nerve fibers. (80).

Neurofilament Protein (NFP):

Neurofilament proteins (NF) form the neurofilaments, which, together with neurotubules, constitute the cytoskeleton of the neurons. Neurofilament cytoskeleton matures during development and shows an upregulation during late embryonic stages and after birth (80,81).

The neuronal intermediate filament protein peripherin is expressed in developing and differentiated neurons from birth up to adulthood. Peripherin stains ganglion cells (76,82).

Microtubule-associated Proteins (MAP):

Microtubule-associated protein 5 (MAP 5) and microtubule-associated tau protein (tau) were excellent markers of the ENS since they were specifically located in nerve cell bodies and nervous processes of normal intestine as well as aganglionic segments.

MAP- 2 stains only ganglion cells without labelling normal or abnormal neural elements (83,84).

Neural Cell Adhesion Molecule:

Neural cell adhesion molecule (NCAM) is a cell-surface glycoprotein involved in cell–cell adhesion during development. It is involved in adhesion between several types of neural cells and their processes and the formation of initial contacts between nerve and muscle. Strong NCAM activity is found in the submucousal and myenteric nerve plexuses, normal nerve bundles and abnormal nerve bundles in HD patients and in the abundant nerve fibers within the longitudinal and circular muscle layers. In contrast, in the aganglionic colon, NCAM activity is either absent or markedly decreased within both the circular and longitudinal muscles (80) .

Nerve Growth Factor Receptor:

Nerve growth factor receptor (NGFR) is the transmembrane protein that binds nerve growth factor (NGF) and brings it into the cell. NGFR immunostaining of normal colon demonstrates numerous NGFR-positive nerve fibers in the circular and longitudinal muscle layers and strong NGFR staining of submucosal and myenteric ganglia. NGFR also stains the hypertrophic nerve bundles in sections from aganglionic colon (80) .

Bcl2:

In colon biopsies of patients with different bowel dysmotility syndromes, Bcl2 was found to be the best biomarker to discriminate immature small neurons in the diagnosis of hypoganglionosis and IND since it was clearly expressed in immature small ganglion cells but did not stain, or only faintly stained, mature ganglion cells (80).

S-100 Protein:

S-100 proteins belong to a large subfamily of calcium binding proteins which are evident in the cytoplasm and nucleus within several nervous and non-nervous tissues. The expression of S-100 proteins has been demonstrated mostly in the glial cells and Schwann cells of the enteric plexus. Thus, S-100 immunohistochemistry displays ganglion cells as prominent negatively stained cells surrounded by immunopositive Schwann cells. S-100 also stains all the nerve bundles (74,76,78,79,85) .

Glial Fibrillary Acidic Protein (GFAP):

Supportive cells of the ENS express glial fibrillary acidic protein (GFAP). GFAP immunoreactivity occurs predominantly in association with the myenteric plexus and to a lesser extent with the submucosal plexus of healthy colon. It has been suggested that the myenteric glia share the astroglial character of the central nervous system. The extrinsic, hypertrophic nerve fasciculi of aganglionic bowel are also immunostained with GFAP (80) .

Synaptophysin*:*

Synaptophysin is an integral membrane protein of the synaptic vesicles facing their cytoplasmic surface. This protein is an index of specific neuronal function such as storage and release of neurotransmitters. Synaptophysin stains submucosal ganglion cells.

There is markedly reduced immunoreactivity (i.e. a decreased number of SY-positive synapses) seen in the intestinal smooth muscle layers of transitional and aganglionic bowel segments, whereas immunoreactive synapses are abundant in the smooth muscle layers of ganglionic colon in HD. SY immunoreactivity also shows ganglion cells and hypertrophic nerve trunks clearly. (86–89)

2.7 DIFFERENTIAL DIAGNOSIS / PROBLEMS ENCOUNTERED IN THE DIAGNOSIS OF HD

In the new born with abdominal distension and failure to pass meconium, other causes of intestinal obstruction are considered, namely:

(1) meconium ileus with / without associated cystic fibrosis,

(2) intestinal malformations such as distal ileal and colonic atresia, isolated or occasionally associated with HD, or colorectal duplication,

(3) functional intestinal obstruction resulting from maternal infection, maternal intoxication, or congenital hypothyroidism (16).

(4) ENS anomalies grouped together as chronic intestinal pseudo-obstruction syndromes, and

Some ENS anomalies, with functional bowel obstruction show very similar, if not identical, presenting symptoms to those of HD. Although these conditions are far less common, they are still difficult to distinguish from HD (90). These anomalies can be grouped as chronic intestinal pseudo-obstruction syndromes with a variable spectrum of pathologic features, including intestinal neuronal dysplasia; hypoganglionosis; immature ganglia; absent argyrophil myenteric plexus; internal sphincter achalasia; and smooth muscle anomalies (91). Chronic intestinal pseudo-obstruction syndromes can be divided into myopathic and neuropathic forms, the latter including the microcolon-megacystic-intestinal hypoperistalsis syndrome. While the recto-anal inhibitory reflex may be normal in these disorders, a full thickness rectal biopsy is necessary for a differential diagnosis (92).

In the older children, one needs to differentiate HD from allied disorders such as ultrashort HD, total colonic aganglionosis (TCA), intestinal neuronal dysplasia (IND), habitual constipation and acquired HD / secondary aganglionosis.

i) Ultrashort HD:

It is defined as aganglionosis with the extension up to 1-2 cm above the dentate line which is the shortest form of HD. Thus, a diagnosis of ultrashort HD can be missed if rectal biopsy/ serial biopsies are taken from higher level. Ultrashort HD diagnosed in a first 3-4 months of life can grow up to 5-6 cm during one and half to two years of life because of the caudocranial growth of the rectum (21,93).

ii) Total Colonic Aganglionosis (TCA):

Total colonic aganglionosis (TCA), a relatively uncommon form of HD is defined as aganglionosis extending from the anus to at least the ileocecal valve, but not >50 cm proximal to the ileocecal valve) and total colonic and small bowel aganglionosis, which may involve a very long segment of aganglionosis. It is not yet clear whether TCA merely represents a long form of HD or a different expression of the disease. There are many differences between TCA and other forms of HD, which require explanation if its ubiquitous clinical features are to be understood. Clinically, TCA appears to represent a different spectrum of disease in terms of presentation and difficulties that may be experienced in diagnosis, suggesting a different pathophysiology from the more common forms of HD. There is also some evidence suggesting that instead of being purely congenital, it may represent certain different pathophysiologic mechanisms.

A rectal biopsy with TCA has more or less same histopathology as classical HD. A lower density of the nerve fibers in the muscularis mucosa and lamina propria in a mucosal biopsy from a suspicion of TCA. If a whole colon is available for examination following total resection, the extent of aganglionosis can be determined by AChE on a Swiss roll of the entire segment. The myenteric plexus is classically called “silent” with neither hypertrophic nerve bundles nor ganglion cells, especially proximal to the splenic flexure.

The patients are invariably female and many of these patients seek help later in life. In vivo, a diagnosis of TCA has to be made based on micro colon extending proximally up to ileocecal junction, and leveling biopsies taken at regular intervals which demonstrate complete aganglionosis.

iii) Intestinal Neuronal Dysplasia (IND):

IND is a developmental abnormality of a submucosal plexus. It is characterized by giant ganglion in the submucosa (>8 ganglia). The nerve cells in the giant ganglia are significantly smaller compared with those cells in normal biopsy. IND is normally seen in association with HD in the proximal segment. These patients are seen to progress to adulthood and be associated with chronic constipation since childhood.

iv) HD in Newborn:

The immature plexus in the new born and young infant show neural units which are much smaller and need to be differentiated from collections of lymphocytes and glial cells. However, presence of immature neural units containing the immature ganglion cells are considered as ganglion cells present thus ruling out HD. NSE and S100 are helpful to study the expression for immature ganglion cells.

v) Habitual constipation:

It is a condition wherein the rectum and distal sigmoid are well ganglionated, yet the child is severely constipated and dependent on enemas. It is usually linked to a poor low fiber diet and poor toilet training. In these cases, the biopsy shows normal histology with ganglion cells and may show increased AChE activity.

vi) Acquired HD:

This is a condition of aganglionosis resulting due to destruction of ganglion cells following infection or due to ischemia. This condition may follow hypoxia following attack of neonatal necrotizing enterocolitis or after a technically tight pull through with attendant ischemia.

2.8 SURGICAL PROCEDURES AND THEIR SIGNIFICANCE IN PATHOLOGY

Survival with HD depends on surgical intervention to remove the aganglionic segment and restore the continuity of the gut. There are three major types of surgery currently performed for HD, which can be performed as either single or multiple stage procedures, either by conventional or laparoscopic surgery. That such a variation can exist suggests that no one procedure has clearly demonstrated its superiority over the rest; conversely it could be argued that significant problems are found in all procedures.

A diagrammatic representation of these three procedures, namely Swenson, Duhamel and Soave is shown in Figure 12. In these procedures, a key step is to ensure that the histological diagnosis of HD is confirmed and the proximal level of aganglionic bowel is ascertained; this can be accomplished by inspecting the bowel (where a transition between ganglionic dilated and aganglionic constructed bowel can often be seen) and by confirming the same with doughnut/ seromuscular biopsies from the proposed margin for urgent intraoperative frozen section, where aganglionic or ganglionic myenteric plexus can be seen.

2.8.1 Swenson's Technique

The first successful definitive surgery for HD was performed by Swenson (94). The sigmoid colon and rectum are mobilised and resected down to the anal canal via a trans-abdominal route. The anal canal is then everted (Figure 12 A) and an anastomosis performed. This procedure involves extensive pelvic dissection that may

severely damage the autonomic nervous supply to both bowel and bladder, the ureters and vas deferens in the male, unless the plane of dissection is kept extremely close to the bowel wall. Concerns about such damage and the subsequent long-term outcome led to the development of other surgical techniques.

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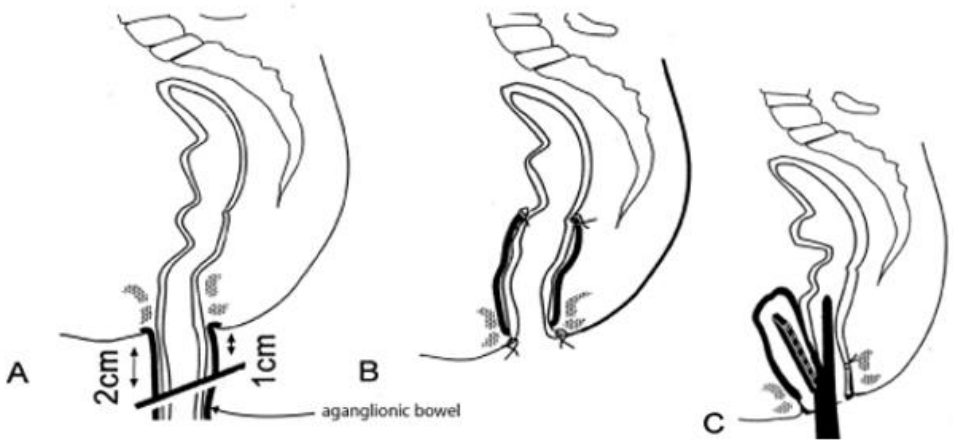

Fig 12: Surgical treatment for Hirschsprung Disease. (A) Swenson; (B) Soave; (C) Duhamel. (95)

2.8.2 Duhamel(Duhamel-Grobb) procedure

In this procedure introduced in 1956 (96) named after Duhamel, the internal anal sphincter is left intact and the only pelvic dissection is performed in the retrorectal space, preserving the innervation of the pelvic organs. The rectum is divided and oversewn at the peritoneal reflection, and the residual aganglionic bowel mobilised and resected. The retrorectal space is then dissected and the ganglionic bowel pulled inferiorly as shown in Figure 12 C. Subsequent modifications have aimed to minimise the blind-ending rectal pouch that may form (97) and introduced surgical stapling devices for the rectal anastomosis (98).

2.8.3 Soave endorectal pull through

Historically, the Soave endorectal pull-through was the last major surgical innovation in the treatment of HD before the introduction of minimally invasive techniques. In 1964, Soave described a submucosal dissection of the aganglionic bowel in the pelvis, thereby preserving the innervations and organs of the pelvis (Figure 12 B) (99). Recently, the Soave procedure has been modified to perform the endorectal dissection entirely transanally (100,101). The abdominal part of the procedure can also be performed laparoscopically (102) or even transanally, resulting in a totally transanal procedure (102). A transanal procedure is chosen only if the radiologic features suggest a rectosigmoid HD.

The resected specimens are processed as per the protocol described under types of biopsy.

2.9 TREATMENT OUTCOME

Surgery has become the routine therapy for infants suffering from HD. But, the functional outcome of surgery is variable. All the standard definitive procedures described above have near comparable long term results with specific variations. The prognosis of the predominant RSHD is predictable and good, the longer forms have a poorer quality of life. When principles are ignored, the long-term consequences of HD, or of repeated surgeries, can be a lifetime of gastrointestinal problems including constipation, faecal incontinence and enterocolitis. This has sparked significant interest for alternative therapies such as stem cell therapy.

Dr Lincoln Stamp at the Murdoch Institute and Ryo Hotta developed techniques for isolating neural stem cells from the gut of embryonic and early post-natal mice and growing neurospheres.

Professor John Furness, devised a method for implanting the neurospheres into the colons of wild-type mice. They were astonished to find that neurosphere-derived cells had migrated a significant distance over the next three weeks.

Electrophysiology experiments performed by Dr Jaime Foong in the Departments of Anatomy and Cell Biology and Physiology showed that the neurosphere-derived neurons fired action potentials and received inputs.

Dr. Gariepy, principal investigator in the Center for Molecular and Human Genetics, has found that stem cells for the enteric nervous system exist in the intestine in an animal model of Hirschsprung disease and that these stem cells can be isolated and transplanted to the gut after birth where they can form neurons. Her team is now working on techniques to harness these stem cells to treat individuals with Hirschsprung disease and other disorders of the enteric nervous system (103,104).

2.10 PATHOPHYSIOLOGY OF HIRSCHSPRUNG DISEASE

2.10.1 The Enteric Nervous System

The enteric nervous system (ENS), the second brain, is the largest and the most complex division of the peripheral nervous system, and is found embedded in the wall of the gastrointestinal tract. In humans, it contains around 500 million neurons and can direct the functions of the digestive system without relying on commands from the central nervous system.

The enteric nervous system of the tubular digestive tract (esophagus, stomach, and intestines) is formed of many interconnected networks, or plexuses, of neurons, their axons, and enteric glial cells (105). Each plexus is composed of enteric ganglia (a group of small cluster of enteric nerve cells and supporting glial cells), which are interconnected by nerve fiber bundles. The processes of these nerve cells connect with

other neurons and innervate the smooth muscle cells that are responsible for gastrointestinal motility. The primary targets of ENS are mucosal secretory cells; gastrointestinal neuroendocrine cells; the gastrointestinal microvasculature that maintains mucosal blood flow during intestinal secretion; and the immunomodulatory and inflammatory cells of the gut that are involved in mucosal immunologic, allergic and inflammatory responses (106,107). The ENS, influences these effector systems in the gut directly or indirectly through its action on intermediate cells, which include neuroendocrine cells, Interstitial cells of Cajal, and cells of immune system (108).

The ENS is made up of an extrinsic and intrinsic component. The extrinsic component consists of a parasympathetic and sympathetic division. The parasympathetic innervation via the vagus nerves controls the motor and secretomotor function of the upper gastrointestinal tract, and via the sacral nerves regulates the functions of the distal colon and rectum. The sympathetic adrenergic fibers from the prevertebral ganglia are responsible for secretomotor neurons containing vasoactive intestinal polypeptide, presynaptic cholinergic nerve endings, submucosal blood vessels, and the sphincters of the gastrointestinal tract (109). The intrinsic component consists of myenteric plexus (Auerbach's plexus) that is seen between the inner circular and outer longitudinal muscle layers, and two layers of submucous plexuses (Fig. 13). The outer submucous plexus is seen adjacent to the circular muscle layer and the inner submucous plexus (Meissner's plexus) is seen close to the muscularis mucosae (105). The myenteric plexus primarily provides motor innervation to the two muscle layers and secretomotor innervation to the mucosa. The submucous plexuses innervate the muscularis mucosa, intestinal neuroendocrine cells, glandular epithelium and submucosal blood vessels. The coordinated activity of multiple neurons residing in

numerous ganglia of both plexus in addition to interstitial cells of Cajal, is required for normal ENS function.

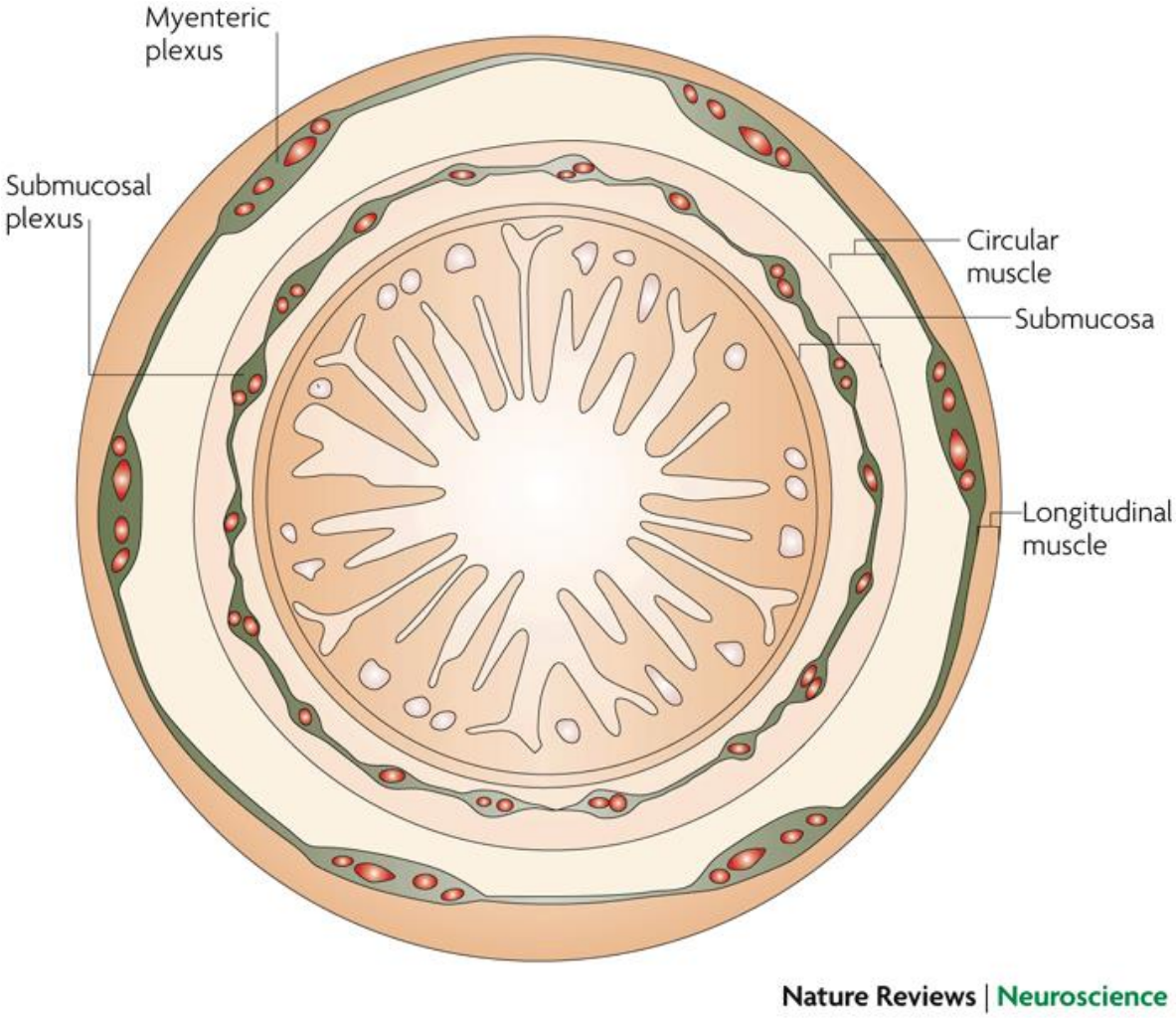

Fig. 13 Schematic representation of a transverse section through the small intestine. Enteric neurons are organized in ganglia (green) found within two main plexuses. An outer myenteric plexus develops first and occupies a position between the longitudinal and circular muscle layers. An inner submucosal plexus forms later in gestation and resides within the submucosa (110).

Glial cells are an integral component of the ENS, and they out number enteric neurons (111). Enteric glial cells resemble the astrocytes of the central nervous system, and their laminar extensions cover most of the surface of enteric neuronal cell bodies. Glial cells permit cell bodies and processes of neurons to be orderly and maintained in proper spatial arrangement and are essential in the maintenance of basic physiological functions of neurons. Furthermore, enteric glial cells produce interleukins and express MHC class II antigens in response to stimulation by cytokines. This suggests a role in modulating inflammatory responses in the intestine (108,112).

2.10.1.1 ENS origin and development

Migratory precursors of the ENS:

The embryonic neural crest arises in the neural tube, originating with the central nervous system, but neural crest cells (NCC) detach from this tissue via reduction of cell-cell and cell-matrix adhesion. The epitheliomesenchymal transformation allows NCC to migrate along pathways of defined routes to various tissues, where they stop moving and differentiate into various cell types (113). The neurons of the ENS are, therefore, derived from the neural crest cells of the embryonal ectoderm (106,107,114,115). This same group of cells also give rise to melanocytes, the adrenal medulla and the sympathetic and parasympathetic ganglia. Any gene mutations that result in a disruption to the neural cell crest cell migration may affect one or all these tissues (116).

Proliferation and Differentiation of the Embryonic ENS:

Neural crest cells migrate along pre-defined pathways to colonize the gut wall as early as 5th week of gestation and are first seen in the developing esophagus. They continue their migration in a craniocaudal direction, during the 5th and 12th week of gestation, down to the anal canal (Fig 14). The first plexus to be formed is the myenteric plexus,

just outside the circular muscle layer. The mesenchymally derived longitudinal muscle layer then forms, sandwiching the myenteric plexus after it has been formed in the 12th week of gestation. Then the submucousal plexuses are formed, by centripetal migration of neuroblasts from the myenteric plexus across the circular muscle layer into the submucosa; again, this too progresses in a craniocaudal direction during the 12th–16th week of gestation (108). Several days after neural crest cells have colonized the gut, these cells are evenly distributed with no indication of cell clustering, except in caecum. As the gut later increases in length and diameter the cells are seen to form ganglia (117). Roman et al. (118) have shown that the nitrergic neurons in human are randomly distributed at 14th week of gestation, and later aggregate into ganglia by week 19.

It is generally accepted that the enteric ganglion cells are derived primarily from the vagal NCC (Fig 14) (106,107,115). Failure of the vagal-derived neural crest cells to colonize the hindgut results in failure of hindgut ENS development, suggesting that interaction between sacral and vagal enteric neural crest cells may be necessary for sacral neural crest cell contribution to the ENS (119). The current belief is that, the ENS is primarily derived from cells of the vagal segment of the neural crest (42,119).

Classically, development of the human ENS has been characterized by the early appearance (between 9 and 12 weeks of gestation) of adrenergic and cholinergic nerves. The development of non-adrenergic, noncholinergic (NANC) or nitrergic innervation is seen during the 12th week of gestation within the myenteric ganglia. Nitrergic innervation in the submucous plexus becomes evident after 14 weeks. Thus, the onset and pace of development of nitrergic innervation are similar to adrenergic and cholinergic innervation (120).

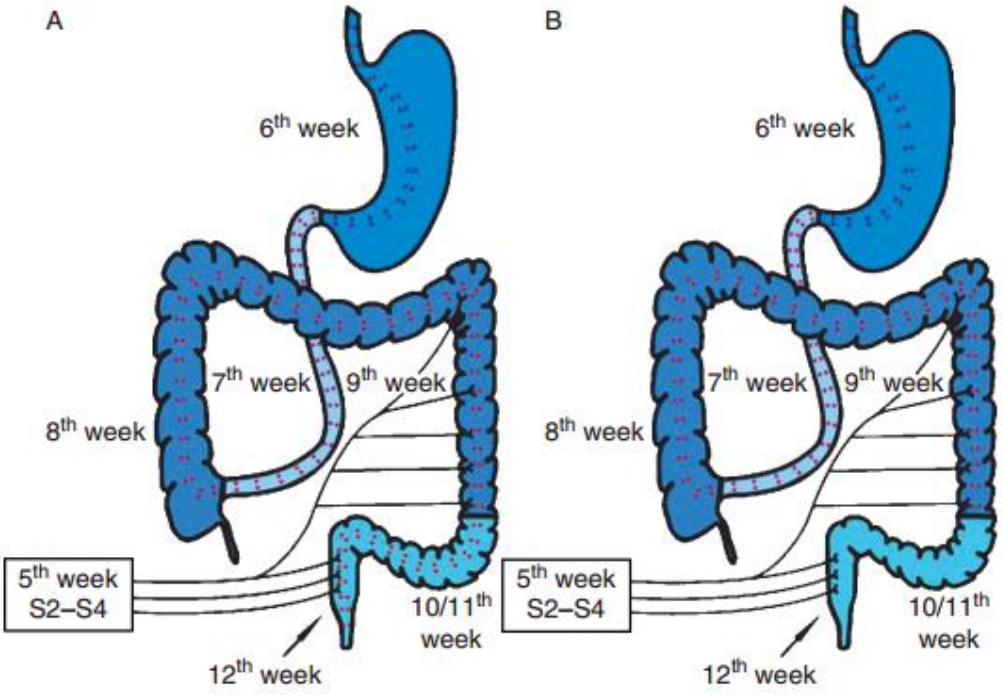

Fig 14. The intramural enteric nervous system develops from neural crest cells (NCCs) predominantly arising from the vagal neural crest. During the 7th week of gestational age, the first enteric NCCs are visible in the upper gastrointestinal tract and jejunum, and subsequently migrate distally to reach the rectum at week 12 (A). The submucosal plexus develops through migration of NCCs from the myenteric plexus, through the inner muscular layer into the submucosa. The extramural innervation consists of the sympathetic fibers from the ganglion mesentericum inferior. In addition, the distal colon (rectum, sigmoid, and a variable distance up to the splenic flexure) receives parasympathetic input from sacral nerves. In Hirschsprung disease (HD), the craniocaudal migration of NCCs is incomplete over a variable segment of the distal hindgut mostly in rectosigmoid (B). The acetylcholine (ACh) output remains unmodulated, and the high release of ACh induces increased acetylcholinesterase (AChE) activity. The increased AChE activity is used as an additional diagnostic tool in short-segment HD. The distribution of the parasympathetic extramural input explains why AChE staining is normal in aganglionic bowel proximal to the splenic flexure. (51)

2.10.1.2 Factors influencing the normal development of the ENS and its defect resulting in HD

The various factors influencing the development of ENS is best explained in a mouse model as shown in the picture below (Fig. 15)

Several receptors have been identified that control the morphogenesis and differentiation of the ENS (120). One of these receptors, RET (REarrange during Transfection) with tyrosine kinase activity, is involved in the development of enteric ganglia derived from vagal neural crest cells (120). The importance of RET in mammalian organogenesis has been further illustrated by the generation of RET knockout mice (121). These mice exhibit total intestinal aganglionosis and renal agenesis. Mutations of RET account for 50% of familial and 15–20% of sporadic Hirschsprung disease (122,123).

Glial cell line derived neurotrophic factor (GDNF) stimulates the proliferation and survival of neural crest derived precursor cells in the embryonic gut (124–126). It has been reported that GDNF is the ligand of RET (127). Mice carrying homozygous null mutation in GDNF show lack of kidneys and ENS, confirming the crucial role of GDNF in the development of the ENS (128,129). Although, a causative role for GDNF mutations in some patients with HD has been suggested, the occurrence of such cases is uncommon, and it is more likely that the GDNF mutations are involved in modulation of the HD phenotype via its interaction with other susceptibility loci such as RET (130,131). The endothelins (EDN1, EDN2 and EDN3) are intercellular local messengers that act via cell surface receptors, EDNRA and EDNRB (132). EDN is initially produced as an inactive preproendothelin that undergoes two proteolytic steps to produce an active peptide. EDN3 and EDNRB have a role in the migration and development of the ENS (133–135). Aganglionosis is seen in mice in which the EDN3 or EDNRB gene was disrupted. Several reports suggest that the downregulation of EDN3 expression may play a role in the pathogenesis of Hirschsprung disease in the sporadic cases (123,136,137).

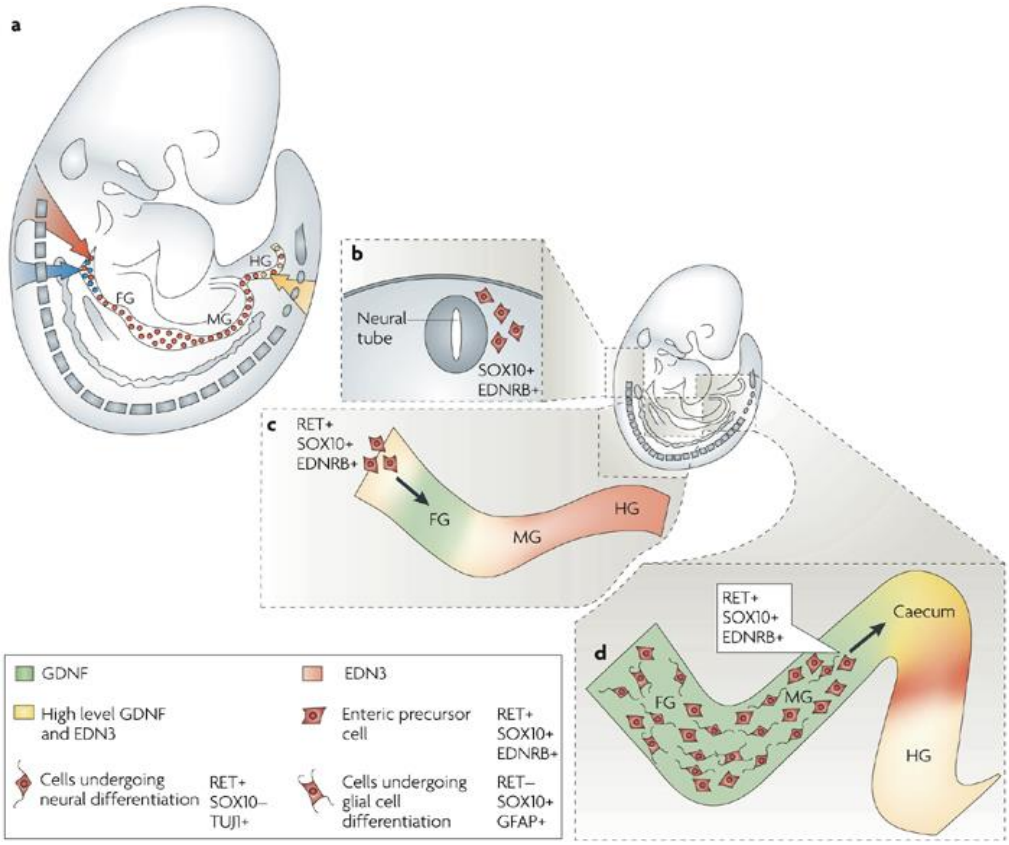

Fig 15. Sources, migratory routes and gene expression in neural crest cells contributing to the ENS.(110)

a | At approximately embryonic day (E) 8.5–9 in the mouse, vagal neural crest cells (red arrow) invade the anterior foregut and migrate in a rostral to caudal direction to colonize the entire foregut (FG), midgut (MG), caecum, and hindgut (HG) and give rise to the majority of the enteric nervous system (ENS, red dots). Colonization is complete by E15.5. The most caudal vagal neural crest cells, emanating from a region overlapping with the most anterior trunk neural crest cells (blue arrow), make a small contribution to the ENS of the oesophagus and the anterior stomach (blue dots). Finally, sacral neural crest cells (yellow arrow) also make a small contribution, beginning their migration at approximately E13.5 and migrating in a caudal to rostral direction to colonize the colon (yellow dots). **b** | As vagal neural crest cells (red) emigrate from the neural tube, they express SRY-box 10 (SOX10) and endothelin receptor B (EDNRB). **c** | Upon entering the foregut at E9–9.5, enteric neural crest-derived cells (NCCs) begin to express RET. Within the gut mesenchyme, the RET ligand glial cell-line-derived neurotrophic factor (GDNF) is expressed at high levels in the stomach (green) and the EDNRB ligand endothelin 3 (EDN3) is expressed in the midgut and hindgut (pink). **d** | As NCCs migrate caudally at approximately E11, they encounter high levels of GDNF and EDN3 expression in the caecum (yellow). Cells behind the wavefront begin progressive differentiation towards neural and glial cell fates. Beginning at E11.5, GDNF and EDN3 are expressed in the distal hindgut.

SOX10 (sex determining region Y-box) gene is expressed in neuronal crest derivates that contribute to the formation of the peripheral nervous system during embryogenesis (138,139). The involvement of SOX10 in the development of enteric neurons was demonstrated in the Dom (dominant megacolon) mouse model of Hirschsprung disease which exhibit distal intestinal aganglionosis (139). Mutations in SOX10 have been identified as a cause of the dominant megacolon mouse and Waardenberg-Shah syndrome in human, both of which include defects in the ENS and pigmentation abnormalities (140,141).

Phox2B gene is a homeodomain-containing transcription factor that is involved in neurogenesis and regulates RET expression in mice. Disruption of Phox2B gene results in a HD like phenotype in these mice (142). Hox11L1 is a homebox gene involved in the development of peripheral nervous system and plays a role in the proliferation or differentiation of neural crest cell lines. The Hox11L1 knockout mouse models (homozygous mutant mice) developed megacolon between 3–5 weeks of age (143,144). Kit, another receptor with tyrosine kinase activity, is involved in the development of the interstitial cells of Cajal (145). Interstitial cells of Cajal are non-neuronal cells that serve as pacemaker cells and are responsible for the spontaneous, rhythmic, electrical excitatory activity of the gastrointestinal smooth muscle. Mice with mutations in the KIT gene lack interstitial cells of Cajal and have changes in skin pigment and abnormal intestinal motility (145). Though no such mutations are so far documented in human disorders, disturbed expression of interstitial cells of Cajal has been reported in patients with motility disorders (146–148).

2.10.2 Molecular Genetics of HD:

The Enteric Nervous System (ENS) is the largest part of the peripheral nervous system and is composed by a network of neurons and glia within the wall of the bowel. Formation of the mammalian ENS requires extensive cell migration, controlled cell proliferation, regulated differentiation, directed neurite growth, and establishment of a network of interconnected neurons. To control the migration, proliferation, differentiation and survival of neural crest derived cells, molecular genetic analysis has identified several genes [Figure 16] [Table2]. Each of those complex cellular events must be guided by coordinated and balanced molecular signals, with a major role of some specific pathways such as the RET/GFRα1/GDNF and EDNRB/EDN3/ ECE1, some transcription factors as SOX10, PAX3, PHOX2B or ZFHX1B, or even some morphogens as SHH, netrins or semaphorins among others. These signaling pathways play a critical role in the formation of enteric ganglia. Failure of this integration leads to the absence of ganglion cells and therefore HD phenotype (149,150) .

HD is frequently associated with other anomalies, and many embryos with multiple defects might die in utero. Most of the HD have a genetic basis and that HD is a complex multigene disorder characterized by incomplete penetrance and variable associated anomalies (53). Mutations in more than 11 different genes have been implicated in pathogenesis of HD (Table3); many were first recognized in murine models for this condition. However, according to most estimates, mutations in one or more of these genes only can be detected inn about half of all HD cases. Therefore, other genetics and / or environmental factors are likely to be involved. RET is the gene in which mutations are most frequently detected in patients with non-syndromic HD. In addition, studies have also indicated that non-coding polymorphisms (base-

pair differences that do not affect protein structure and which are present in >1% of the normal population) in the proximal portion of RET gene pose a significant risk for HD, possibly by reducing RET expression (151–153).

Table2: Hirschsprung disease associated genes and their clinical features (110).

Genes	Position	Inheritance	Phenotype
RET	10q11.2	Dominant, incomplete penetrance	Non-syndromic MEN2A
GDNF	5P12-13.1	Non-Mendelian	Non-syndromic
EDNRB	13q22	Recessive Dominant (de novo in 80%)	Shah-Waardenburg Non-syndromic
EDN-3	20q13.2	Recessive Dominant, incomplete penetrance	Shah-Waardenburg Non-syndromic
SOX10	22q13.1	Dominant (de novo in 75%)	Shah-Waardenburg
NTN	19p13	Non-Mendelian	Non-syndromic
ECE-1	1p36.1	Dominant (de novo)	Congenital heart formation
PHOX2B	4p12	Dominant (de novo in 90%)	Haddad Syndrome (CCHS)
ZFHX1B (SIP1)	2q22	Dominant (de novo)	Mowat-Wilson
KIAA1279 (KBP)	10q22.1	Recessive	Goldberg-Shprintzen
TTF1 (TITF1)	14q13	-	Non-syndromic
NRG1	8p21	-	Non-syndromic

Table 3: Susceptibility genes in isolated and syndromic HD(53)

SYNDROME	GENETIC DEFECT	% WITH HD	COMMON PHENOTYPIC FEATURES
Isolated HD[a]	RET EDNRB[b] EDN3[b]	100%	
	TTF-1	NE	TTF-1 mutations have been associated with hypothyroidism and cleft palate, but the only reported HD patient with a TTF-1 mutation and isolated HD.
Syndromic HD			
MEN2A	RET[c]	<1% (154)	Medullary thyroid carcinoma, pheochromocytoma, parathyroid hyperplasia.
Smith-Lemli-Optiz	DHCR7	16%(154)	Growth retardation, pedal syndactyly, mental retardation, hypospadias, dysmorphic facies.
Down	Trisomy 21	2-9%(154)	Prominent epicanthal folds, upslanting palpebrae, hypotonia, mental retardation, flat midface, single transverse palmar crease.
Waardenburg-Shah	SOX10 EDN3[b] EDNRB[b]	100%[d]	Deafness, piebaldism, other neurological deficits.
Mowat-Wilson	ZHFX1B	62%(155)	Abnormal facies, cardiac malformations, mental retardation, genitourinary anomalies.
Haddad	PHOX2B[e]	100%[f]	Congenital central hypoventilation, neuroblastoma.
Goldberg-Shprintzen	KIAA1279	100%	Microcephaly, various brain malformation, cleft palate.
X-linked hydrocephalus	LICAM	NE	Cerebral aqueductal stenosis, hydrocephalus, absent corpus allosum

Cartilage-Hair Hypoplasia[g]	RMRP	NE	Skeletal dysplasia, sparse blond hair, immunodeficiency, anemia.
Bardet-Biedl	BBS1, BBS2, BBS4, BBS6[h], BBS7, BBS8	2%(156)	Obesity, retinal degeneration, polydactyly, gonadal and renal malformation
Kauffman-McKusick	MKKS[h]	NE	Polydactyly, congenital heart defect, hydrometrocolpos.

[a]Since HD most often present shortly after birth features of some syndromes (e.g., MEN2A) may not be obvious at the time of diagnosis.
[b]Isolated HD is more commonly associated with heterozygous mutations; Waardenburg-Shah syndrome is more commonly associated with mutation in both alleles.
[c]Missense mutations affecting one of three cysteine codons (Cys 609, Cys 618 or Cys 620).
[d]HD is a diagnostic feature that differentiates Waardenburg-Shah syndrome from other variants of waadernburg syndrome.
[e]Congenital central hypoventilation syndrome has also been associated with RET, GDNF, and EDN3 mutations in rare patients, but concurrent HD has only been associated PHOX2B mutations.
[f]HD is a diagnostic features of Haddad syndrome, but PHOX2B mutations also occur in patients with isolated hypoplasia.
[h]BBS6 and MKKS are the same gene
NE, not established

2.10.2.1 Genes involved in the development of HD

The genetic dissection of HD has led to the identification of 12 genes and five susceptibility loci implicated in the development of HD disease. These 12 genes are expressed either in the enteric neural crest cells (NCCs) or in other cells in the gut along the path of NCCs migration during ENS development.

The inheritance of HD is complex. The disease can be transmitted as a dominant trait or as a recessive trait, but in most cases, it is probably polygenic with differences in

sex ratio and a male predominance in RSHD (4:1) Incomplete penetrance and variable expression, associations with many syndromes and congenital malformations have all been observed (16). Linkage analyses of multiplex HD families revealed that the *RET* (REarrange during Transfection) gene, located at 10q11.2, is the major risk factor for Hirschsprung disease as almost all families showed linkage with *RET* (157–159). Coding sequence mutations in *RET* are responsible for a dominant form of HD (with incomplete penetrance) and coding and splice site mutations have been identified in up to 50% of familial cases and 15-35% of sporadic cases (160). The mutations are scattered throughout the *RET* coding sequence, including large and micro-deletions and a variety of point mutations. *RET* mutations associated with HD are believed to cause a loss of function (haplo-insufficiency) (161–163). However, *RET* mutations on their own might not result in aganglionosis, as the penetrance of the *RET* mutations (in general) is 72% in males and 51% in females. Yet *RET* is believed to be the major gene underlying HD, as evidenced primarily in families enriched for LSHD (159). Mutations in the rest of the 11 genes namely, *EDNRB* (164), *EDN*3 (165,166), *GDNF* (131,167), *NTN* (168), *SOX10* (140), *PHOX2B* (140), *ECE1* (140), *KIAA1279/KBP* (140), *ZFHX1B* (169,170), *TTF-1* (171) and *NRG1* (171) do not exceed 20% of the cases, supporting a genetic heterogeneity for this disorder. Finally, rare heterozygous germline mutations of *EDNRB* in combination with *RET* germline mutations have also been detected in patients showing the HD phenotype (171). This supports the concept of synergistic heterozygosity for HD, i.e. that the disease phenotype could be the result of the cumulative effect of at least two mutations in different genes.

Mutations in most of the 11 genes are found mostly in the syndromic cases where HD is associated with other congenital malformation (see Table2). Mutations in *EDNRB*, *EDN3* and *SOX10* were identified in a patient with Shah-Waardenburg syndrome

(WS4), which is characterized by congenital hearing loss, pigmentary abnormalities of the hair, skin and eyes, and HD disease (172,173). Mutations in *PHOX2B* have been identified in patients with congenital central hypoventilation (CCHS) and HD disease. This syndrome is also known as Haddad syndrome. CCHS is a rare disorder characterized by impairment of autonomic control of spontaneous respiration in the absence of other lung or cardiac disease (174). Mutations in *ZFHX1B* have been identified in patients with Mowat-Wilson syndrome, which is an autosomal dominant disorder characterized by mental retardation, epilepsy, delayed motor development, and HD disease (174). Mutations in *KIAA1279* (now called kinesin-binding protein or *KBP*) have been identified in patients with Goldberg-Shprinten (GOSH) syndrome, a rare autosomal recessive disorder characterized by HD, microcephaly, mental retardation, and polymicrogyria (175). A mutation in *ECE1* was identified in a single patient with craniofacial and cardiac defects (176).

Besides mutations in these 11 genes, chromosomal abnormalities are frequently observed and found in 12% of all syndromic HD cases. Trisomy chromosome 21 (Down Syndrome (DS)) is the most frequent chromosomal abnormality (>90% cases) associated with HD disease. On the other hand, 77% of DS patients have associated gastrointestinal abnormalities, of which 2-15% can be explained by HD disease (177). This might implicate chromosome 21 in the etiology of HD, so it is not surprising that DS patients have a 40-fold higher chance of getting HD compared to the normal population (178). The other possible explanation could be that the genetic imbalance disturbs the homeostasis of genes expressed during development that eventually contribute to the disease phenotype (179). Interestingly, besides these possibilities, a variant in the *RET* gene also seems to be related to the HD phenotype in DS patients. It was shown that a common single nucleotide polymorphism (SNP), rs2435357

(C>T), located in the conserved region of intron 1 of the *RET* gene, when homozygous for the T-allele, gives DS patients a 5.3-fold increased risk of developing HD compared to DS patients who are homozygous for the wild-type C-allele (180). Furthermore, the frequencies of the T-allele differ significantly between individuals with DS alone (0.26±0.04), HD alone (0.61±0.04) and those with HD and DS (0.41±0.04), showing there is association and genetic interaction between RET and the chromosome 21 gene dosage (178).

2.10.2.2 Signaling Pathways and their interactions:

Two signaling pathways play a very important role in ENS development; the intracellular signaling pathways downstream of GDNF/RET-GFRα1 signaling pathway and the EDNRB/EDN3 signaling pathway mediated by EDN 3 ligand and its G -protein -coupled receptor EDNRB which play a role in enteric neural crest derived cells (NCCs) survival, proliferation and migration as well as in coordinating normal colonization of the gut by NCCs.

2.10.2.2.1 GDNF/RET-GFRα1 Signaling Pathway

RET (REarrange during Transfection) is a transmembrane tyrosine kinase receptor that can bind four different ligands: GDNF, NTN, artemin and persephin. These ligands do not bind directly to the extracellular domain of RET. Instead, they bind to co-receptors inserted in the membrane via a glycosyl-phosphatidyl-inositol (GPI) group: GFRα1, GFRα2, GFRα3, and GFRα4 respectively (181).

GDNF is a secreted protein and a distant member of the TGF-β superfamily. GDNF binds to the glycosyl-phosphatidyl-insoitol linked receptor, GFRα1. The GDNF-GFRα1 complex then binds to and activates the transmembrane receptor tyrosine kinase, RET [Figure 16 and 17]. Mutations in genes encoding members of the GDNF/RET-GFRα1 signaling pathway account for about 50% of familial

cases and around 30% of sporadic cases of HD. Non-coding mutations in RET have also been proposed to increase susceptibility to HD. In mice, *Gdnf* is expressed by the gut mesenchyme prior to the entry of neural crest cells. *Ret* is expressed exclusively by neural crest-derived cells and *Gfrα1* is expressed by both crest-derived cells and the gut mesenchyme. *Gdnf*-, *Gfrα1*- or *Ret*-null mice die within 24 hours of birth, and lack enteric neurons along the entire length of the gastrointestinal tract caudal to the stomach. *Gdnf*+/- and *Ret*+/- mice are viable and do not exhibit aganglionosis.

RET is subject to alternative splicing and translated into two functional isoforms, RET51 and RET9, which differ in the number of amino acids at their C terminal end. These isoforms are highly conserved between human and mouse (23,110,182). Mice lacking the Ret51 isoform (*Ret9/9* mice) have enteric neurons along the entire length of the gastrointestinal tract, while mice lacking the Ret9 isoform (*Ret51/51* mice) suffer colonic aganglionosis (183). The phenotype of the *Ret51/51* mice is highly reminiscent of the colonic aganglionosis observed in patients with HD. Interestingly, the developing ENS in humans appears to be more sensitive to reduced RET signaling than that of the ENS in mice. *RET* mutations in human act dominantly to give rise to HD, whereas ENS development is normal in *Ret* heterozygous mice (184).

The endothelin family has three members: EDN1, EDN2 and EDN3. These molecules are 21 amino acid peptides that binds to seven transmembrane domain G-protein-coupled receptors. Two types of receptors have been described in mammals: EDNRA, which binds preferentially to EDN1, and EDNRB, which binds all three peptides with the same affinity. The EDN1/ EDNDRA pathway is

involved in the ontogeny of cranial and cardiac NCC, whereas the EDN3/EDNRB pathway is required for the development of melanocytes and the ENS (181).

2.10.2.2.2 EDN3/EDNRB Signaling Pathway

Endothelin 3 (ET-3) is a secreted peptide, which is expressed by the gut mesenchyme. ET-3 is initially expressed in an immature form before being processed to an active peptide by the enzyme, endothelin converting enzyme 1 (ECE1). ET-3 signals through the receptor Endothelin Receptor B (EDNRB), which is expressed on migrating enteric neural crest cells [Figure 16 & 17].

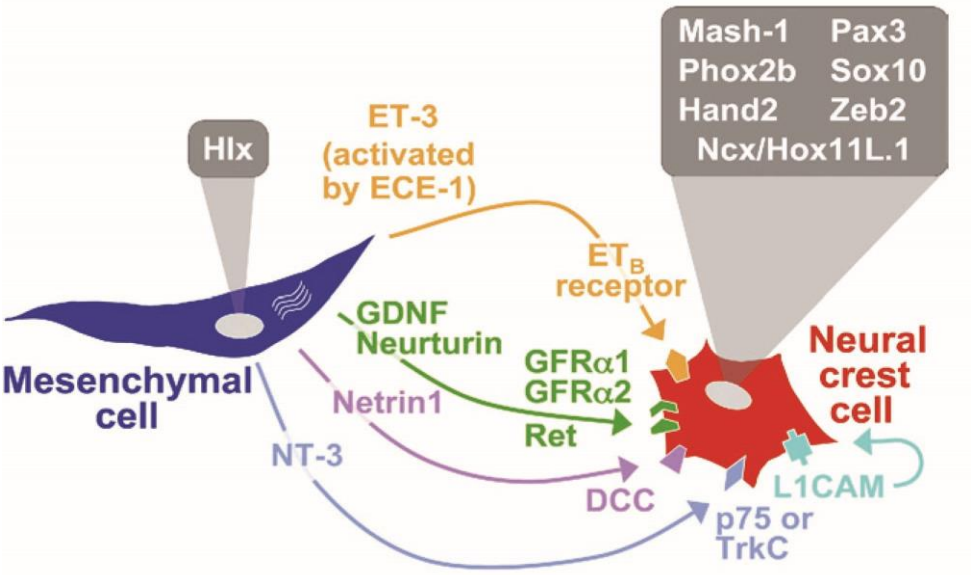

Fig 16: Signalling pathways and transcription factors known to be required for development of the mammalian enteric nervous system. Signalling molecules synthesized by intestinal mesenchymal cells and their receptors are shown in colour; the transcription factors found in intestinal mesenchymal cells and in neural crest cells are shown in the grey boxes. ET-3, endothelin-3; ECE-1, endothelin converting enzyme-1; ETB receptor, endothelin-B receptor; GDNF, glial cell line-derived neurotrophic factor; GFR, GDNF family receptor; DDC, *deleted in colon cancer* gene product; NT-3, neurotrophin-3. (182)

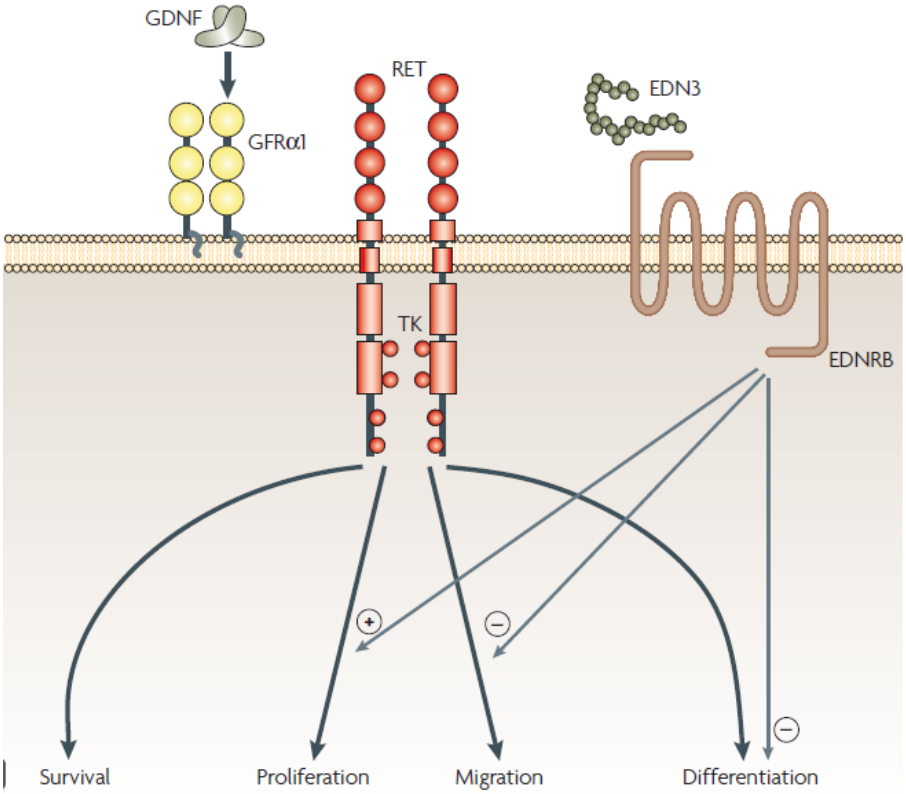

Fig 17. Signalling through the RET and EDNRB receptors regulates aspects of ENS development. Enteric neural crest-derived cells (NCCs) express the RET receptor tyrosine kinase (TK), which is activated upon dimerization following the formation of a complex with dimers of its ligand glial cell-line-derived neurotrophic factor (GDNF) and the glycosylphosphatidylinositol-anchored co-receptor GDNF family receptor α1 (GFRα1). Activated phosphotyrosines are represented by small circles in the intracellular region. NCCs also express the G-protein coupled-receptor endothelin receptor B (EDNRB), which is activated upon binding of endothelin 3 (EDN3). RET signalling is implicated in promoting (+) the survival, proliferation, migration and differentiation of neurons in the enteric nervous system (ENS). Signalling through EDNRB has been implicated in maintaining NCCs in a proliferative state and inhibiting (–) differentiation. (110)

Mutations in *ET3* and *EDNRB* account for around 5% of HD cases. *ET3*- and *EDNRB*-associated HD can present as both syndromic (such as Wardenburg-Shah syndrome) and non-syndromic forms of HD. In mice, *lethal spotted* (*ls*) and *piebald lethal* (*sl*) are naturally occurring mutants of *Et-3* and *Ednrb* respectively, and lack enteric neurons in the distal bowel. As with *RET*, the human ENS appears to be more sensitive to reduced *EDNRB* signaling than that in mice.

(23,110,182) .Around 21% of patients heterozygous for the W276C mutation in *EDNRB* develop HD (185), while heterozygous *piebald lethal* (*sl*) mice do not develop any form of aganglionosis. (186)

2.10.2.3 Interactions between the GDNF/RET and EDN3/EDNRB pathways

Genome wide association studies have revealed associations between the inheritance of EDNRB mutations and certain RET alleles in patients with HD. This joint transmission of EDNRB and RET alleles in patients with HD suggests a genetic relationship between these two loci. This idea is supported by genetic studies in the mouse. Studies in mice, using a two-locus complementation approach, confirmed a genetic interaction between the *Ret* and *Ednrb* loci by showing that the generation of *Ret+/-*; *Ednrbs/s* mice resulted in colonic aganglionosis; a phenotype not observed in *Ret+/-* or *Ednrbs/s* mice alone. (187,188) A similar genetic interaction was also reported using *Ret51* and *Et-3ls* mice. A significant increase in aganglionosis, extending all the way to the stomach, was observed in *Ret51/51*; *Et-3ls/ls* mice compared to the colonic aganglionosis normally observed in *Ret51/51* or *Et-3ls/ls* mice alone. The mechanism underlying these interactions is not yet known; however, it has been proposed that *Ret* and *Ednrb* may interact by activating common downstream signaling molecules, such as protein kinase A (189) and also Sox 10.

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AIMS & OBJECTIVES

1. To study the biology of Hirschsprung disease in the Indian subpopulation by characterizing its demography and pathomorphology, the latter comprising of histological, cytochemical and immunohistochemical features of the enteric nervous system.
2. To correlate RET gene in Hirschsprung disease with the length of aganglionosis.

MATERIAL & METHODS:

4.1 General

SOURCE OF DATA:

Samples (Tissue- fresh, formalin fixed; slides and blocks for review and EDTA Blood) were received from children with suspect Hirschsprung disease from St. John's Medical College Hospital and from other institutions/hospitals with referral to the Department of Pathology for diagnosis.

Study area: Department of Pathology**,** St. John's Medical College, Sarjapur Road, Bangalore- a national referral center for the diagnosis of Hirschsprung disease

Study design: Prospective cross-sectional observational study.

Study period: Nov 2011 to June 2015

IERB Clearance: Ethical clearance obtained from the Institutional Ethical Review Board. (vide IERB Ref study no 201/2011)

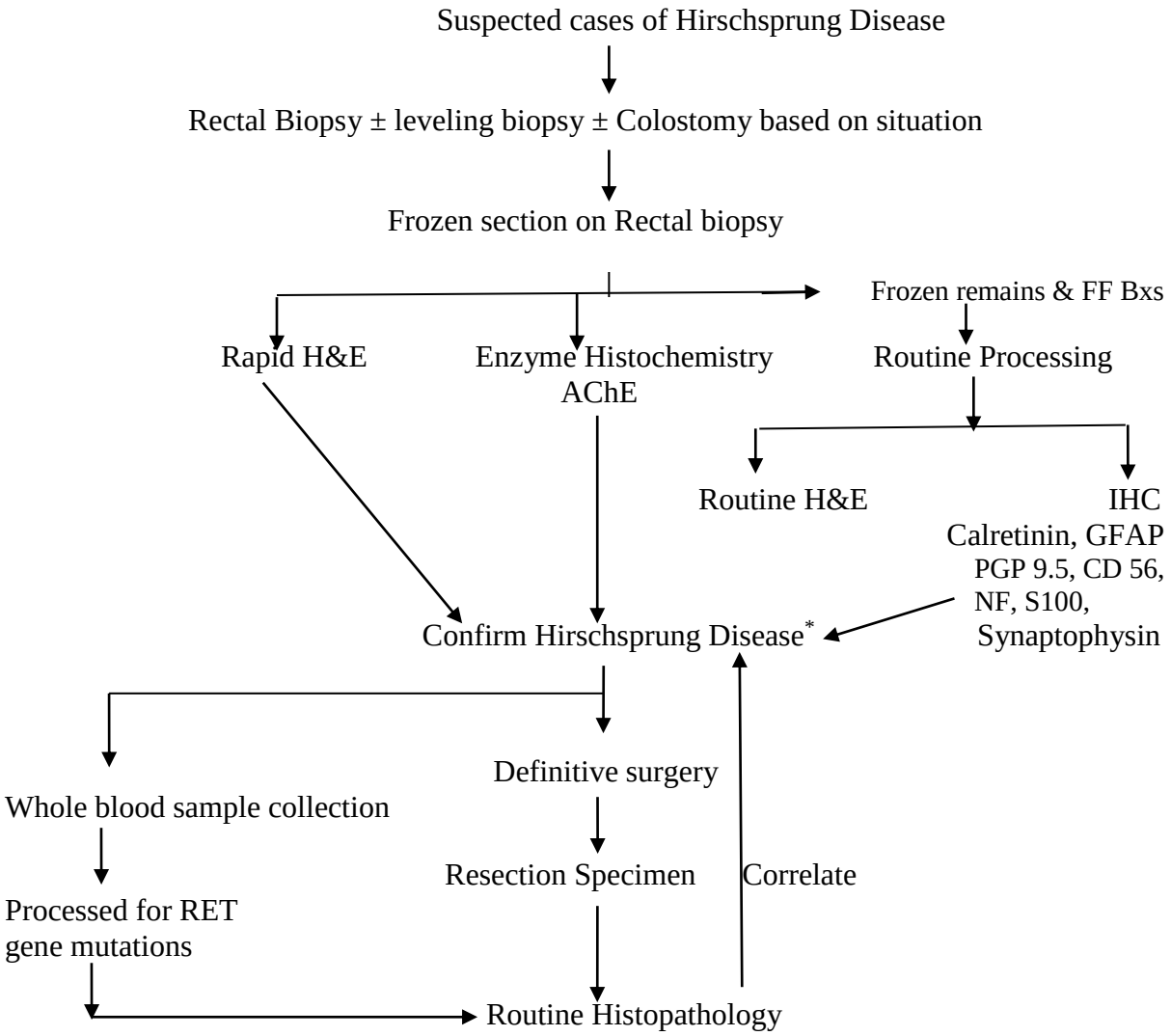
METHODOLOGY

Subjects:

Paediatric cases with a provisional clinical diagnosis of Hirschsprung Disease (HD) fulfilling the prescribed selection criteria listed below (suspected HD) were included in the study. The diagnosis was confirmed /refuted by histology, enzyme histochemistry and immunohistochemistry as shown in the flowchart below.

Methodology

Biology of Hirschsprung disease:



Abbreviations used:

Bxs -Biopsies, H&E – Haematoxylin and Eosin, AChE– Acetylecholine Esterase,

IHC -Immunohistochemistry, SDH – Succinate Dehydrogenase, FF- Formalin Fixed.

* Those biopsies negative for Hirschsprung disease were considered as non-Hirschsprung disease (NHD).

Inclusion Criteria:

- ❖ Patients with delayed passage of meconium (>24 hrs).
- ❖ Patients with intestinal obstruction and clinical features compatible with Hirschsprung Disease (HD).
- ❖ Patients with constipation dating back to infancy.

Exclusion Criteria:

- ❖ Patients with surgical (e.g., anal fissure) or medical (hyperthyroidism) etiology or constipation.
- ❖ Habitual constipation responsive to diet/ laxative/ enema schedule.
- ❖ Inappropriate/ inadequate biopsies such as-
  a) Minute biopsies from anal canal showing anal epithelium
  b) Biopsies in which the submucosal zone is mostly occupied by lymphoid follicles.

The samples from children were all those referred to Department of Pathology for histopathologic diagnosis. The study is approved by the Institutional ethical committee [APPENDIX- I- A, B and C]. Parental informed consent for participation in this study was obtained for all specimens (EDTA Blood sample/ colorectal biopsies) obtained [APPENDIX- 1I and III].

and

Fresh Biopsies

Rectal biopsies (mucosubmucosal) were taken 2-3 cm above the dentate line. Biopsies taken at laparotomy were seromuscular/ full thickness. These biopsies were collected fresh in 0.9% saline soaked gauze piece, were grossed and then embedded on the OCT freezing medium which is frozen to form a platform of 0.5

cms on the chuck using Leitz cryostat. The tissue was then kept for freezing for a minimum time of 5 minutes at temperature -22^{0}C to -25^{0}C.

Sections were cut at 10 µ using LEICA CM1510 cryostat and were routinely stained with

a) Rapid Haematoxylin & Eosin (H & E stain) for checking adequacy by assessing the amount of submucosal tissue in the tissue sample and [APPENDIX- IV]

b) Rapid improvised Acetylcholinesterase (AChE) stain, on all biopsies (mucosal /full thickness rectal, full thickness circumferential bowel - doughnut) in cases of HD and additionally ileal and appendicular biopsies in cases of suspect TCA for highlighting acetylcholinesterase activity in the submucosa and mucosa [APPENDIX- V].

Formalin fixed Biopsies

Biopsies collected in 10% buffered neutral formalin are processed to make Formalin fixed paraffin embedded blocks (FFPE). These FFPE tissues were cut at 3-5 µ and were stained with routine H&E stain and with a panel of immunohistochemistry using Calretinin, GFAP, Synaptophysin, PGP 9.5, CD 56, NF and S 100 markers to highlight the enteric nerve plexus and abnormal nerve fibres and study their distribution [APPENDIX- VI].

Blood Samples for Molecular studies:

Peripheral blood samples were collected from the diagnosed cases of Hirschsprung disease in EDTA vacutainer for the genetic (RET mutation) study. Consent from all patients and controls for molecular analyses were obtained.

Blood sample as control (15 in number) were obtained from patients diagnosed as non- HD. Genomic DNA was extracted from 2 ml of EDTA blood sample using QIAamp DNA mini kit (Qiagen, CA, USA) [APPENDIX- VII]. The extracted DNA was examined by electrophoresis and the yield was measured spectrophotometrically using BioSpec- nano spectrophotometer for life science/ Qubit 2.0 Fluorometer before use. PCR amplification of template DNA was primed by a template melting setup at 94^0C for 5 min followed by 35 cycles of serial incubation at 94^0C for 1 min, 60°C to 67°C annealing for 3 min and 72°C extension with a final extension step of 5-8 minutes in an Eppendorf master cycler gradient. The exons are amplified from genomic DNA by use of primers and reaction conditions standardized in our lab. Genotypes were determined by digestion of the PCR product by EXO SAP- IT enzyme method and electrophoresis on an agarose gel. EXO SAP- IT (EXO- nuclease I; Shrimp Alkaline Phosphatase) removes unused primers and nucleotides. The results were further analyzed by DNA sequencing analysis by use of the Sanger's sequencer.

The selected patients were followed up for a period of six months.

All the subjects enrolled in the study were blinded for confidentiality and given a unique identification number. Relevant clinical and histopathological details like age/sex of the subject, phenotype of HD, associated anomalies, sporadic/ familial status and follow-up data were collected from the patients immediate relative/ records.

STATISTICAL ANALYSIS

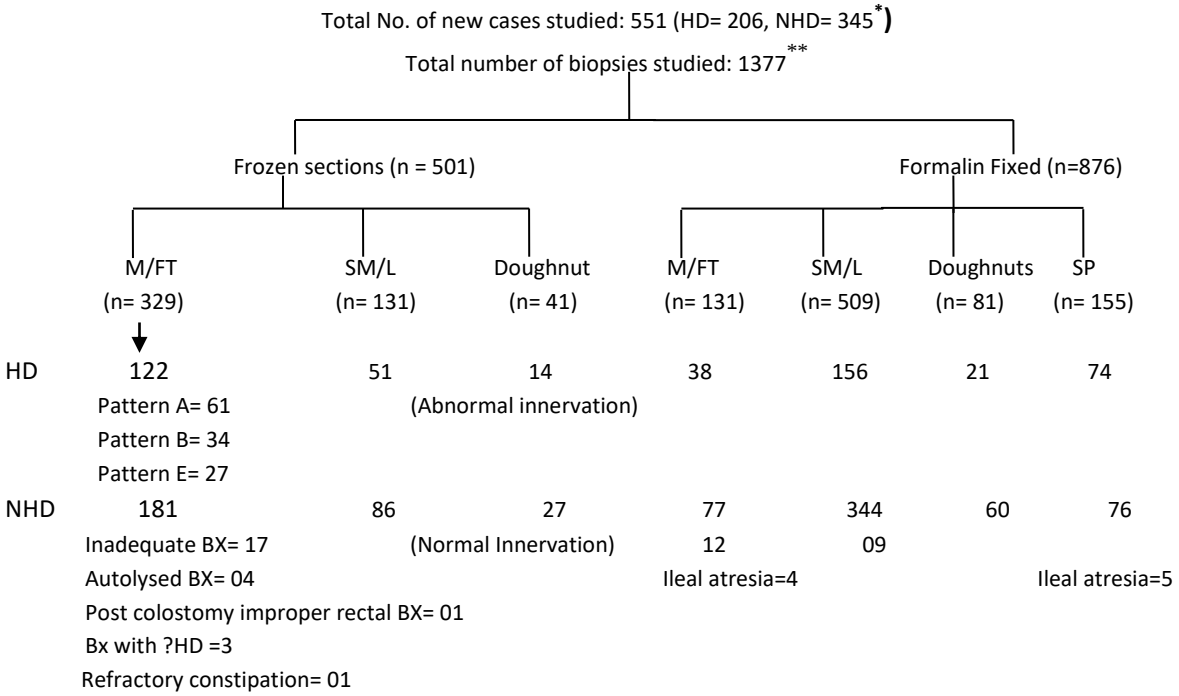
The data from the studies were included in to a statistical software and analysis was carried out using SPSS 16 and 20 (SPSS Inc, Chicago, IL). $P < 0.05$ was considered as significant. Descriptive analysis and cross tabs were performed

using Chi- square test and Cohen's κ coefficient was used to assess inter observer agreement between two groups wherever required.

4.2 Samples studied during the study period

A total number of 1377 biopsies from 551 clinically suspect cases of HD were studied at this centre from January 2012 to June 2015. Fresh mucosal, seromuscular, and doughnuts for frozen sections constituted 501 while the rest (876) were resected specimens that were received in formalin.

The fresh biopsies were studied by rapid H&E and Toluidine Blue (wherever required) and AChE stains were performed on all rectal biopsies when received fresh and on doughnuts whenever abnormal innervation was suspected on H&E. The immunohistochemistry panel of neural markers were carried out in relevant cases. The The distribution of these cases is shown below.



* 2 cases mimicked HD. One was diagnosed as Internal anal sphincter achalasia and other as chronic pseudo intestinal obstruction.

**Each case has more than one biopsy (See Table 1 below)

Abbreviations used:

NHD= Non Hirschsprung disease, M= Mucosal, FT= Full thickness, SM= Seromuscular, L= Leveling,

SP= Resected specimen

Table 1: Number of biopsies in HD cases

No. of biopsies received	No. of HD cases
1	234
2	74
3	43
4	54
5	30
≥6	34

Of the 1377 biopsies, 206 were newly diagnosed cases of HD. The diagnosis of HD was made on a single biopsy in 234, while 74 had 2, 43 had 3, 54 had 4, 30 had 5 and 34 had more than 6 biopsies for diagnosis and management, Pattern A and B of AChE staining was seen in 61 and 34 respectively, whereas the equivocal pattern with hypertrophic nerve bundle was seen in 27.

345 cases were confirmed as non-HD; if an emergency blind/non-levelled colostomy had been done in such cases, their stomas were closed. 9 of these cases had ileal atresia and one had refractory constipation.

The routine diagnostic work on cases of suspected HD helped in fine tuning the enzyme histochemistry and immunohistochemistry protocols and execution of the subsequent five research studies namely, Study 1 to Study 5.

RESEARCH WORK: Study 1 to 5

STUDY -1

TITLE

Improvised double-embedding technique of minute biopsies: A mega boon to histopathology laboratory:

BACKGROUND

The optimal orientation of minute biopsies during paraffin block embedding facilitate visualization of the mucosal architecture in assessing villous morphology in malabsorption syndrome or neural plexii in well-defined tissue planes for definite tissue diagnosis of Hirschsprung disease (HD). The difficulty is amplified further when these minute biopsies are from neonates. In gastroenterological units, the mucosal biopsies are placed on thin cut cardboard/thick paper pieces with mucosa facing upwards. This latter technique greatly facilitates orientation and is widely practiced.[1] Yet, bowel biopsies in suspect aganglionosis often reach the laboratory irregularly curled up, both when sent fresh in saline or in formalin. The time spent in reorientation is a waste of the pathologist's time and energy.

Regardless of the state on reaching the laboratory (fresh/fixed) or the fixatives used, it is necessary to precisely orient mucosal biopsies. The most suitable material for tissue embedding is the bacteriological agar.[2,3] We aimed to evaluate a modified technique of agar embedding of minute biopsies. We have examined a method of orienting and embedding fresh tissue initially in optimal cutting temperature (OCT) medium for frozen section to demonstrate acetyl cholinesterase activity and thereafter re-embedded the frozen tissue

remains in agar. In tissue sent in a fixative like formalin, we have embedded first in agar before further tissue processing and paraffin wax embedding. This technique of agar embedding of minute biopsies seems simple, efficient and user-friendly.

MATERIAL AND METHODS

Mucosal biopsies, received fresh or fixed in formalin, were examined with a magnifying glass to orient them and their measurements in greatest and smallest dimensions noted. The embedding surfaces were identified by noting the velvety, pale pink mucosa and the adjacent hemorrhagic submucosa. These were kept dry, oriented perpendicular to the cutting surface on a filter paper and that surface was marked gently by surgical coloring inks from Histolab/acrylic paint from Fevicryl acrylic hobby color groups (no. 6, 12, 22, 33)[4] using tip of a paper strip. Seromuscular bowel biopsy was identified as a C-shaped pale, translucent firm structure; this ‘c’ was considered as the embedding surface. This is a very important step in biopsy orientation as practiced during frozen section and the C-shape of the fragment occurring due to contracture of smooth muscle especially the muscularis propria, is taken advantage for the proper orientation of mucosal biopsies with attached muscle, especially when the fragment is not sent fixed on paper. When leveling biopsies were received from various sites of the colon, that closest to the anus was colored green, followed by yellow, blue and India ink sequentially. Thus, more than one colored fresh tissue was embedded in OCT medium in one block [Figure 1a], mimicking a microarray block for cutting frozen sections after documenting the color against the origin of the tissue sample. The cut frozen sections were stained with rapid Hematoxylin and Eosin (H and E) as well as processed for enzyme histochemistry. After the frozen sections were reported, the OCT embedding medium was allowed to thaw; the inked tissue washed lightly with water to take away OCT remains and re-embedded in the next medium, namely agar as described below [Figure 1a-c].

3% of bacteriological agar (SISCO Research Lab, extra pure bactograde Lab Reagent) in 10 mL of distilled water was prepared in a beaker by gradually bringing to boil on a hot plate with continuous stirring. This solution was slowly cooled to room temperature and poured into the steel embedding moulds. To hasten the gelling process, the moulds were kept in the freezer/ cryostat for 1-2 min. If not, the agar solidifies in about 8-10 min at room temperature. When it is solidified, four punch holes were made for the four biopsies in one block using punch biopsy forceps of diameter 1-6 mm depending on the size of the biopsy. The inked biopsies which were already oriented on the filter paper were gently lifted with fine forceps and placed into the punch holes in the agar block with their embedding surface facing down. The other three biopsies were similarly embedded in the agar sequentially. Molten agar was poured on top of the tissue in the punch hole to fill the cavity and seal it [Figure 1b, inset].

After the agar was set, the agar block containing the embedded tissue was easily detached from the embedding moulds and the edges cut to ensure that 3 mm of agar was left all around the tissue. All the tissue samples thus embedded were in a single plane, that is, the embedding surface of the agar block was the embedding plane for all the tissues. Subsequently, this agar block was placed inside a disposable cassette and processed in a histokinette using the regular cycle used for other tissues. After the last phase of impregnation, the agar block containing the four different colored tissues was ready for embedding with their embedding surfaces facing down into the molten paraffin. This double preembedded agar-paraffin block [Figure 1c] was allowed to cool on a cold plate after which it was ready for serial sectioning at 4 μ. They were cut in the same manner as the routine paraffin embedded blocks. The sections, thus, obtained were either stained with H and E [Figures 1c and 2a] or taken on coated slides for immunohistochemistry.

We have also processed other biopsy samples (e.g., corneal discs after penetrating keratoplasty, endobronchial biopsies, soft tissues biopsies, core biopsies, circumferential ring bowel biopsies etc.) similarly.

At the end of the technique, all the biopsies were evaluated for the following features:

- Ease of embedding and cutting sections.
- Degree of tissue processing.
- Numbers of paraffin blocks used.
- Microscopic tissue orientation and least number of sections necessary for proper assessment.
- Interference of coloring inks in the gross and microscopic interpretation

RESULT

One hundred and two mucosal biopsies from sites such as colon, cornea, conjunctiva, vocal cord and bronchus ranging in size from 0.2 to 0.5 cm were processed by this improvised double embedding technique over a study period of 2 months in the histopathology laboratory attached to a tertiary teaching medical college hospital. The technique went through a series of improvisations till it was well standardized, made user friendly and acceptable by technical staff and yielded excellent results to be employed on a routine basis in the laboratory.

Conventionally, it is always difficult to orient minute mucosal tissues as small as 0.2 cm because of the reduction of the tissue volume and surface area to near half after formalin fixation and paraffin processing. In such situations, it is difficult to identify the embedding surface during embedding as the coloring inks invariably color the entire tissue and not the embedding surface alone. The preembedded agar-paraffin blocking technique has overcome

this issue as the tissue when fresh would have already been embedded in agar before processing. The results of this technique have been evaluated and are listed below:

Ease of embedding and cutting sections

The technique has helped in optimally orienting biopsies as small as 0.2 cm. We were able to embed easily such stained tissues in agar at a convenient time. They were processed along with the other routine tissue blocks and embedded with other blocks in paraffin by any technical staff. We did not need an experienced staff to do this exercise. As the agar was colorless and transparent in liquid, gel and solid forms, the orientation of the biopsy could be easily viewed/monitored with naked eye or through a dissection microscope and stained with coloring inks as applicable. The agar paraffin blocks were easy to cut and were well suited for cutting serial sections of thickness as thin as 2-3 μ. No technical difficulties were experienced while cutting sections. The sections were easy to spread as the agar did not shrink appreciably during processing and were least subjected for folds and because of smooth cut, resulted in no holes/chatters, provided there were no trapped air bubbles while preparing the blocks. The colored inks remained permanently on the tissues in the blocks [Figure 1c]. This helped in easy identification of the various tissues especially when there were >1 tissue in a block and helped to make sure all the fragments were trimmed well so as to get complete sections.

We encountered one problem during orientation. If tissues were minute and were not held down during embedding in agar, they could float and come up to get placed themselves at different levels in the agar block making it difficult for all tissue fragments to be placed in a single plane. As a result, tissue fragments did not appear as complete cuts in a single section at any given time. In such situations, the block had to be cut carefully at different levels to get to have all tissue fragments; this resulted in more work, loss of time and undue delay. We

tried to overcome this problem by gently holding down the tissue by mounting needle till the agar poured into the punch hole over the fragment solidified. When there were >1 tissue to be embedded, the tissue needed to be held down for every fragment. This care taken was worth the effort at the end of the exercise. This problem could also be overcome by embedding on a glass slide if the tissues are too small as described by Ventura et al[34]. The agar is poured on a glass slide and allowed to gel and then tissue is placed into the gel as described above. The process is faster by this method as the agar used is less. The thin plate of agar with inked tissues embedded in it on a glass slide as described above, is lifted up with a blade, edges cut and re-embedded in a larger agar block as described above. As the height of the agar block is thin and as the fragments inked can be easily visualized with ease, this improvisation was a great help to get the right plane for minute tissues during embedding.

Assessment of degree of tissue processing

These agar-paraffin embedded tissues were found to be well processed, firm and securely held and hence the agar as a preembedding medium for tissues inside had not interfered with the penetration of chemical reagents while processing. It was also noted that the tissues in the agar medium had shown minimal shrinkage in their sizes after processing and no loss at any time. In situations, were the fragmented biopsies were too small to be picked up, the fluid (saline/formalin) with the tissue fragments is spun at 1000 rpm for 30 s. The fluid is decanted, a drop of dye added to the sediment and the entire deposit is embedded in-toto into a well-made in the solidifying agar on a glass slide as discussed above. Thus, even the smallest fragment though not visible with naked eye in agar block was identifiable microscopically as the tissue was inked. The tissue in agar and paraffin block remained well preserved like any other routine paraffin block.

Number of blocks used

The number of blocks were cut down drastically during frozen as well as in the paraffin block depending on the type of biopsy received - fresh as for frozen or fixed in formalin. Differential gross coloring of tissue samples and group embedding in OCT medium for frozen section helped in many ways. Instead of cutting one frozen section for each tissue biopsy, one needed to cut only one block at one go with as many as four biopsies in it but coloured differently, thus saving an easy 60 min of turnaround time (TAT) for frozen section (accepted TAT for one frozen block is 20 min as per National Accreditation Board for Testing & Calibration of Laboratories (NABL)). We embedded a maximum of 4 minute biopsies coloured differently in a single agar block without any difficulty provided the biopsies were small and >4 different coloured inks were available.

Microscopic orientation and least number of sections necessary for proper assessment

Since the biopsies were preembedded in agar in a desired orientation and agar embedded tissues were found to be well processed, firm and securely held, the orientation remained as it is through the entire process till the microscopic study. This helped in avoiding deeper sections unnecessarily and therefore, also helped in assessing the biopsies with least number of sections. As agar-paraffin block mimicked a tissue microarray block[34], one single section containing a maximum of four tissue samples from four different sites from the same patient helped in cutting down the number of blocks to be cut, manpower and reagents for special techniques such as enzyme histochemistry and immunohistochemistry. The site of biopsy and the coloring stain used for it, needed to be well documented in the gross description.

Interference of coloring inks in the gross and microscopic interpretation

Surgical coloring inks from Histolab/acrylic paint from Fevicryl acrylic hobby color groups (no. 6, 12, 22, 33) used were excellent inks to color the tissues. The coloring inks helped in tissue identification all through the processing, microscopic examination and forever. We have observed that the inks after drying didn't fade even after several changes in processing solutions. We also found that these inks have never interfered with the stains used for diagnosis, e.g., H and E, histochemical stains, enzyme histochemistry and immunohistochemistry.

We also noted that the agar remained with the tissue all through unlike paraffin. As the agar imbibed minimal/negligible amount of hematoxylin during staining, it remained minimally stained giving a very faint background hue [Figure 2a] which did not interfere with H and E stain [Figure 2a], histochemical stains [Figure 2b, AB-PAS], enzyme histochemistry [Figure 2c] or immunohistochemistry [Figure 2d] and also during photography.

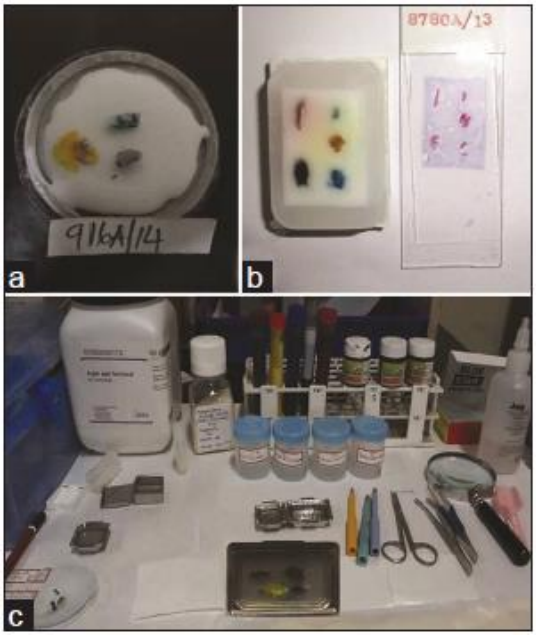


Figure 1: (a) Minute biopsies coloured with different inks embedded in freezing medium for frozen section, (b) Biopsies coloured with different inks embedded in a single block of agar-paraffin and its corresponding section on stained with routine (H and E), (c) Set of materials required for agar embedding technique. Inset: Coloured biopsies embedded in agar medium.

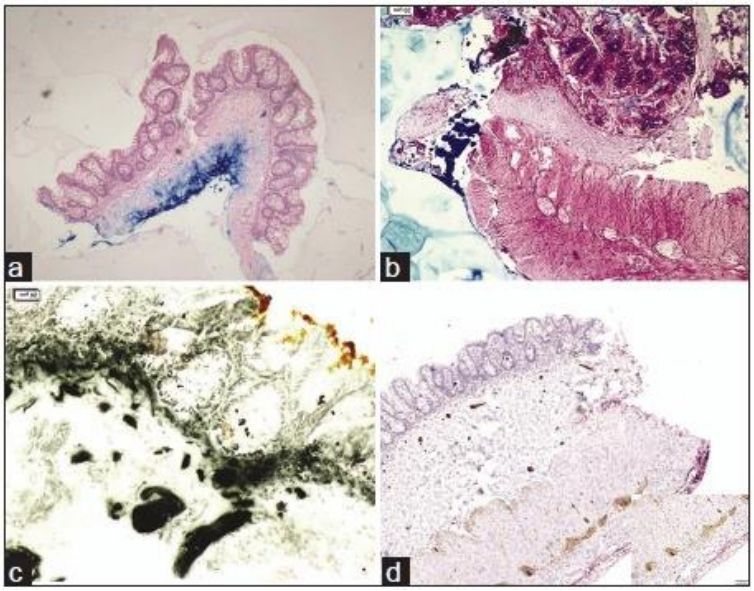


Figure 2: (a) H and E, section (×40) from blue inked biopsy embedded in agar-paraffin. The blue ink has not interfered with the (H and E) (b) AB-PAS section from blue inked biopsy shows AB-PAS positive glands. The blue ink has not interfered with the special stain (×40) (c) AChE histochemistry on rectal mucosa in Hirschsprung disease shows increased activity (black fibres). The yellow ink has not interfered with the enzyme activity (×100) (d) Rectal biopsy (coloured red) embedded in agar and stained for calretinin highlights ganglion cells in myenteric plexus. The red ink has not interfered with the immunostain (×400).

DISCUSSION

The bacteriological agar that is composed of sub units of galactose is extracted from the cell wall of red algae.[6] Agar is best used for tissue embedding because of its property of hysteresis[6,7] that is, it remains solid at 36°C ± 1.5°C, continues to remain firm at 60-65°C (when paraffin wax is molten, thus holding the embedded tissue tight and well oriented) and melting temperature of 87°C ± 1.5°C, a temperature range, which a histokinette never reaches. Thus, the agar remains solid all through the tissue processing and the embedded oriented tissue fragment in agar remains secured and oriented all through the process.

While differentially coloring tissue grossly and embedding one has to make sure that the inked tissue is dry before it is embedded, lest, the ink spreads into the embedding medium. Agar must be poured carefully onto the tissue after it is placed inside the holes in agar. Additionally, the agar must be neither not too cool (jelly like, does not pour) nor too hot (tissue floats and loses its orientation). If the agar fails to enter into the crevices around the tissue, it is insecure, tends to fall out or entrap air pockets. Avoiding air pockets is crucial to hold the tissue securely during further processing and section cutting. One can also use agar gelatin mixture to make the preembedding matrix stronger. [7] Once the sections were cut and rehydrated in a 50-55°C water bath for mounting on to the marked glass slides, the agar became soft allowing the tissue to expand as required for proper mounting onto glass slides with minimal or no folds.

Another advantage was that, during processing, the agar does not hinder in formalin penetrating into fresh tissues embedded in it.[6] Formalin penetrates the agar completely to fix the tissue without disturbing the agar. Thus, this technique of agar-paraffin embedding facilitates tissue fixation, processing and optimal orientation and avoids tissue loss. This

technique may also be ideal for embedding precious tru-cut-biopsies from various organs obtained under image guidance.

To carry out this agar embedding technique on a larger scale, we agree with Ventura *et al*.[5] who have devised a method where in the agar solution could be prepared in large volume, divided as aliquots of 3-5 ml into test tubes and refrigerated. The required number of test tubes could be taken out as required, as per the number of biopsies to be embedded in that sitting and agar is melted either by using a simple water bath or a micro wave at 300 watts for 30 s or 600 watts at 10 s. Setting of the agar would take approximately 8-10 min and the agar blocks can be prepared and kept ready just before the fresh tissue embedding. This is probably an additional step to the routinely practiced tissue embedding technique. Care needs to be taken to see that the agar blocks are prepared fresh and give no scope to become like a culture medium.

We agree that the principle and technique described here for embedding multiple tissues in agar-paraffin block can also be used to prepare a tissue microarray block.[5,8] We have embedded upto four tissue samples in a block. A single section may feature upto four tissue samples from four different sites from a patient and these can be used for special techniques such as immunohistochemistry provided the origin of tissue and coloring stain used is documented. Occasionally, one could embed >4 biopsies, provided they are colored by four different coloring inks and tissue size is small enough to be accommodated in one block. This improvised technique is advisable for precious tru-cut-biopsies obtained by computed tomography and ultrasonography, small skin, ophthalmic and temporal artery specimens.[9] The traditional coloring ink in use is India ink but when different surface/margin/tissue need to be colored, the other permanent color inks from Histolabs or acrylic paint from Fevicryl acrylic hobby color groups (no. 6, 12, 22, 33)[4] may be employed. These inks stain both fresh and formalin fixed tissues, survive the overnight processing and the subsequent staining

procedures on cut sections and are seen clearly in the paraffin blocks as well as microscopically on the periphery of the sections. They do not unduly color the processing fluid or spread within the tissue sections. Unlike inks from Histolabs, these acrylic hobby colors are easily available in most stationary shops, have long shelf life, are non-toxic and can be bought as single units at economical price.[4] Red and magenta inks are preferably avoided as they may get obscured by the eosin of H and E stain at microscopy level.

CONCLUSION:

The modified agar-paraffin embedding technique represents a simple, reliable, user-friendly method that can greatly improve the quality of diagnostic information one can obtain from minute biopsies by improving on tissue orientation, quality of sections and shortened TAT. It also economizes on number of paraffin blocks, manpower, chemical reagents and laboratory infrastructure.

PRESENTATION:

This scientific work was presented (oral) at the 60th Annual Meeting of Society of Paediatric Pathology (SPP) (Jointly with the Society of Paediatric Pathology, SPP) at Birmingham, United Kingdom, 4th-6th September 2014. [APPENDIX-VIII]

PUBLICATION:

This scientific work was published in Indian Journal of Pathology and Microbiology which is as follows:

Yadav L, Thomas S, Kini U. Improvised double embedding technique of minute biopsies: A mega boon to histopathology laboratory. Indian J Pathol Microbiol 2015; 58:12-6.

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STUDY -2

TITLE:

Improvised rapid Acetylcholinesterase histochemistry versus calretinin immunohistochemistry in the evaluation of colorectal biopsies for Hirschsprung disease

BACKGROUND:

Hirschsprung disease (HD), a congenital malformation of the enteric nervous system (ENS) characterized by aganglionosis commonly affects the rectosigmoid region and is a leading cause of neonatal large gut obstruction and childhood constipation. HD affects 1/4500-5500 live births in the West and Japan[1,2] and 1/3000-3500 in Asiatics.[3] Though no published epidemiological data is available from India, a referral center for HD like ours that has screened over 3000 colorectal biopsies for suspect HD over 15 years currently diagnoses about 60 new cases annually.

Despite differences in the opinion across the Atlantic, acetylcholinesterase (AChE) histochemistry is considered to be a reliable method for the intra-operative frozen section diagnosis of HD and the subsequent leveling of aganglionosis. However, the laboratory facility and technical expertise for this exist in few centers across our country. We have trained pediatric surgeons and their pathologists from over 30 state and central tertiary health care centers across India over the last 4 years in annual hands-on workshops; yet only 3/30 institutions have implemented AChE histochemistry routinely for managing their patients. The optimal evaluation of multiple biopsies for diagnosis and leveling can interrupt the workflow of a surgical pathology laboratory, even with the rapid technique. These logistic problems in establishing AChE histochemistry has simultaneously fuelled our interest in immunohistochemistry (IHC) with a battery of markers such as S-100, neuron specific enolase (NSE), C-Kit, PGP 9.5, and synaptophysin.[4] However, none has proved to

be a reliable single marker in clinching the diagnosis of HD on formalin fixed rectal mucosal biopsy.

Calretinin, a vitamin D dependent calcium binding protein expressed in central and peripheral nervous system, has been studied in the ENS too. Absence of calretinin expression in HD was first reported on resected specimens in 2004[5] and subsequently on rectal mucosal biopsies.[6-10] In this study, we have compared calretinin IHC as a primary diagnostic tool in the diagnosis of HD with our standardized improvised rapid method of AChE histochemistry[11-13] for the 1st time in India.

MATERIAL AND METHODS

Material

This cross-sectional comparative study was conducted at the Department of Pathology of a tertiary teaching medical college hospital which serves as a referral center for the diagnosis of HD over a 3-month period. The following were included:

a. All rectal biopsies that were performed to diagnose HD or exclude a clinical diagnosis of HD. They were either fresh/fixed in formalin, adequate (mucosubmucosal)/ inadequate (mucosa with scanty submucosa/totally replaced by lymphoid follicles/low level with anorectal junctional mucosa) and submitted for histological (hematoxylin and eosin [H and E])/histochemical (AChE) evaluation.
b. Colorectal resection specimens obtained at definitive pull through surgery in previously confirmed cases of HD.
c. Colonic, ileal and appendicular biopsies from suspect cases of long segment colonic Hirschsprung disease (LScHD) and total colonic aganglionosis (TCA).

Ethical clearance for this study was obtained from the Institutional Ethical Review Board.

Methods

Frozen sections were cut from fresh rectal biopsies at 10 µ thickness using Leica CM1510 cryostat at different levels. Two sections were stained with rapid H and E and two with AChE by employing our standardized modified rapid method with a staining time of 40 min.[13] A frozen section positive for HD was run as a positive control. The frozen tissue remains were preserved at -25°C for further use if necessary. Where no formalin fixed tissue was available, the frozen tissue remains were embedded in agar medium, fixed in formalin and processed for routine histopathology. In suspect LScHD/TCA, ileal biopsies and the appendix were similarly processed for AChE and thereafter fixed in formalin.

All the formalin fixed, paraffin embedded tissues were cut at 4 µ and stained with H and E. One section of full-thickness biopsies and 12 sections (two slides) of mucosal biopsies were taken. One section per slide was stained for calretinin IHC using Novolink Max Polymer Detection System and ready to use antibody (prediluted) from Dako (Clone-DAK Calret 1) with heat antigen retrieval in ethylene diamine tetra acetic acid(EDTA) buffer at pH 9.0. A full-thickness section of colon with ganglion cells was stained as a positive control while the mast cells in the mucosa were considered as an inbuilt positive control. A negative control involved the omission of the primary antibody. No AChE was attempted on formalin fixed tissue as it is not suitable for AChE enzyme studies. One section each from the ganglionic and aganglionic segment of the resected bowel at pull through was studied with H and E and calretinin staining. The sections thus stained (H and E, AChE and calretinin) were studied independently by two reporting pathologists who were blinded to the clinical details.

A diagnosis of HD or non-Hirschsprung disease (NHD) was made after a consensus between the histopathologists and correlation with patient clinical details. The following diagnostic criteria were employed.

Diagnostic criteria for NHD in a rectal mucosal biopsy:

- At least one ganglion cell is identified in one or more tissue sections and/or
- The AChE stains an occasional nerve twig in the submucosa and highlights a ganglion cell on frozen section of fresh biopsy. However, no stainable AChE fibers are identifiable (negative staining) in the muscularis mucosa (MM) and lamina propria (LP) and/or
- The calretinin immunostain shows distinct linear granular black fibers in the MM and LP, extending upwards to a variable distance in between the crypts (positive staining) and stains ganglion cells (both nuclear and cytoplasmic) in the submucosa if included in the biopsy (formalin fixed) [Figure 1a].

Diagnostic criteria for HD in a rectal mucosal biopsy:

- Absent ganglion cells, presence of hypertrophic nerve bundles in the submucosa and
- Increased AChE activity with positive staining of hypertrophic nerve fibers as dark green- black staining in specific patterns (pattern A — nerve fibers in the submucosa extending through the MM into the LP akin to an arborizing tree trunk, pattern B — nerve fibers extending only up to the base of the crypts, equivocal pattern-hypertrophic nerve bundles in the submucosa alone with no specific pattern in the LP) [Figure 1b] on frozen section of fresh biopsy and /or

- With calretinin IHC, the biopsy (formalin fixed) demonstrates neither ganglion cells nor any stainable fibers in the MM and LP (negative staining).

The collated data were analyzed using SPSS version 16 (SPSS Inc, Chicago, IL) and $P < 0.05$ was considered as significant. Mean and standard deviation were obtained for continuous variables while Cohen's κ coefficient was used to assess agreement between calretinin and the AChE as read by two pathologists.

RESULT:

The test group comprised of 74 paraffin blocks from 51 suspect HD cases studied in addition to three ileal biopsies, three appendices and two circumferential full-thickness ring bowel biopsies (doughnuts) over 3 months. These included 18 frozen remains fixed in formalin from 18 fresh rectal mucosal biopsies after being processed for AChE (18 cases) and 56 biopsies in formalin (33 cases). Seven biopsies were from neonates (2 fresh, 5 in formalin). The study confirmed HD in 26 cases (22 rectosigmoid HD, 1 LScHD and 3 TCA) and NHD in 25.

The control group comprised of full-thickness formalin fixed rectal biopsies from ganglionic and aganglionic segments of 10 pull through segments which formed the negative and positive control group, respectively. The demographic data of the two groups are compared in Table 1.

Table 1: Demographic data of 51 cases in the study

Variable	Control group	Test group
N	10	51
Age		
Mean ± SD	12.09 ± 10.67 months	24.05 ± 37.55 months
Range	3 days - 2 years	1 day – 8 years
Gender n (%)		
Male	10 (100)	43 (84.3)
Female	0 (0)	08 (15.7)

Control group

The H and E sections showed ganglion cell clusters placed at regular intervals in the submucosal and myenteric plexus and no hypertrophic nerve bundles (Figure 1a). The calretinin stain showed distinct intensely granular positive fibers in the MM and mucosa between the crypts, reaching up to the surface lining at foci. Calretinin stained both the nucleus and cytoplasm of ganglion cell clusters along with the extrinsic nerve fibers in the serosa. The positively stained mast cells were seen scattered in all layers of the bowel, but predominantly in the LP.

Test group

The findings in the 74 paraffin blocks from rectal biopsies (18 fresh, 56 formalin fixed) from 51 cases and their eight appendicular/ileal/doughnut biopsies received are discussed separately as follows.

The fresh and formalin fixed rectal biopsies (Figures 1a-c and 2a-c) are described separately below.

Fresh rectal biopsies

Eighteen fresh rectal biopsies (3 full-thicknesses, 15 mucosal) from 18 cases (2 neonates) were studied.

H and E stain

Of 18 fresh rectal biopsies (3 full-thickness, 15 mucosal), 7 were conclusively diagnosed (3/3 full-thickness biopsies — HD (Figure 2a), 4/15 mucosal biopsies — NHD). No diagnoses was possible in 11/15 mucosal biopsies (7-inadequate, 4-low level mucosal biopsies) (Table 2).

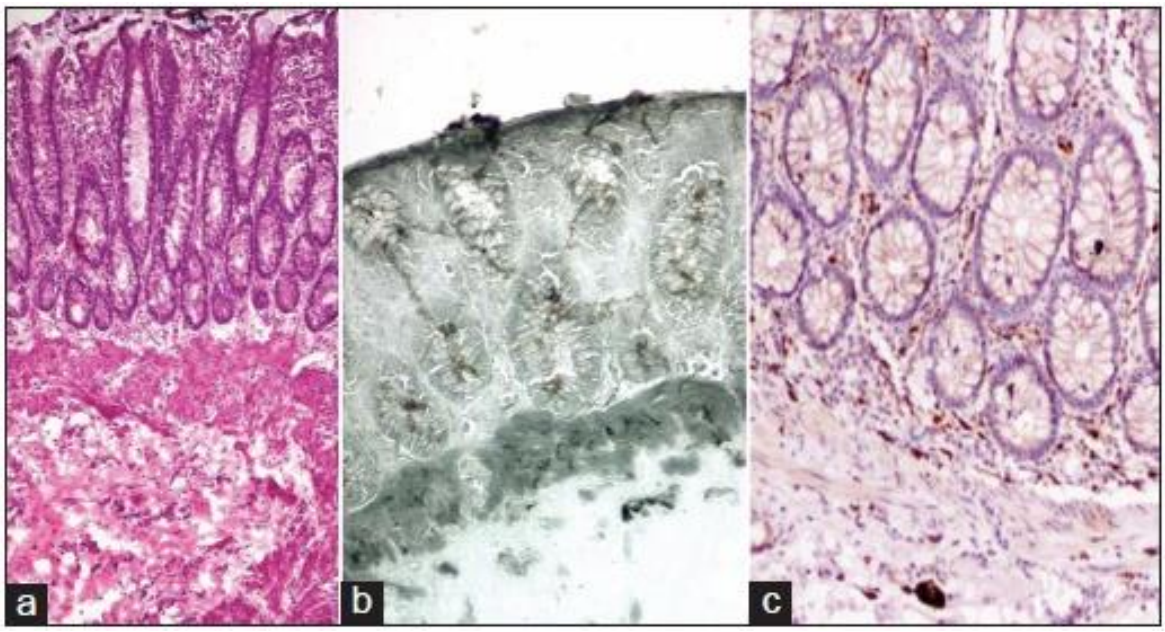

Figure 1: Non-Hirschsprung disease: Normally innervated rectal mucosa (a), showing no increase in Acetylcholinesterase activity (b), and calretinin staining positive fibers in the mucosa and muscularis mucosa (c) (×200).

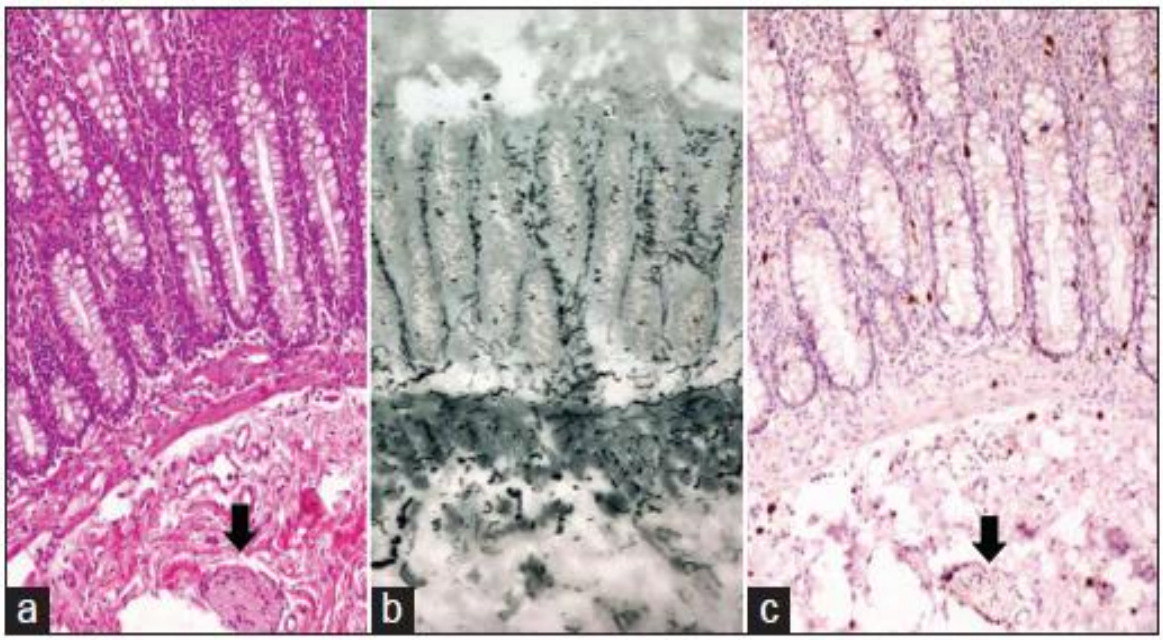

Figure 2: Hirschsprung disease: Rectal mucosa (a) showing hypertrophic nerve bundle in the submucosa, increase in Acetylcholinesterase activity (b) of pattern A and negative staining with calretinin (c) note the negative staining of hypertrophic nerve fiber (arrow) with calretinin.

Table 2: Analysis of fresh rectal biopsies from 18 cases of suspect HD

FRESH RECTAL BIOPSIES (n = 18, c = 18)										
Stain	**FULL THICKNESS n=3**		**MUCOSAL n=15**							
			Adequate n=4		**Inadequate n=7**			**Low level n = 4**		
	HD	**NHD**	**HD**	**NHD**	**HD**	**NHD**	**No diagnosis**	**HD**	**NHD**	**No diagnosis**
H&E	3	0	0	4	0	0	7	0	0	4
AChE	3	0	0	4	2	5	0	1	3	0
Calretinin	3	0	0	4	2	5	0	1	3	0

AChE stain

AChE confirmed HD in 6/18 cases - 3 full-thickness, 3/11 mucosal where diagnosis was not possible with H and E (inadequate-2/7, low level-1/4) (Tables 2 and 3). Of the six, two were neonates with an equivocal pattern and pattern A (Figure 2b) in one each. NHD with an equivocal pattern (Figure 1b) was proved in 11/18 cases; of these 3/11 were 'adequate' mucosal on H and E and 8/11 where no opinion was possible on H and E (5/7-inadequate biopsy, 3/4-low level biopsies). An 8-year-old with onset of constipation since infancy was diagnosed as refractory constipation as the mucosal biopsy showed ganglion cells as well as the increased AChE activity in the mucosa with pattern B.

Table 3: Fresh biopsies: Comparison of sensitivity, specificity, predictive values (positive and negative) and accuracy of calretinin versus AChE in readings by the two pathologists.

Indicators	**First pathologist**		**Second pathologist**	
	AChE (%)	***Calretinin (%)***	**AChE (%)**	***Calretinin (%)***
Number of samples	18	18	18	18
Sensitivity	6/6 (100)	12/12 (100)	6/6 (100)	12/13 (92.31)
Specificity	12/12 (100)		12/12 (100)	
PPV	6/6 (100)	12/12 (100)	6/6 (100)	12/12 (100)
NPV	12/12 (100)	6/6 (100)	12/12 (100)	6/7 (85.72)
Accuracy	18/18 (100)	18/18 (100)	18/18 (100)	17/18 (94.45)

Calretinin stain

The calretinin staining showed positive staining (Figure 1c) in all the 12/18 biopsies diagnosed as NHD by AChE including the three low level biopsies and one of refractory constipation. Six biopsies with no stainable fibers (Figure 2c) (reported by the first pathologist) correlated with increased AChE of HD. Thus, the concordance of the calretinin readings with AChE was excellent with the first pathologist (sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy being 100%). Though the results obtained from the second pathologist were largely agreeing with the first, there was one false-positive diagnosis of HD where the minute, thin fibrillar fibers with calretinin were missed because of a lymphoid follicle obscuring the fibers. The sensitivity, specificity, PPV, NPV and accuracy are tabulated in Table 3. However, the hypertrophic nerve bundles seen in biopsies with HD were not stained with calretinin.

Formalin fixed biopsies

Fifty-six rectal biopsies (43 full-thicknesses, 13 mucosal) from 33 cases (5 neonates) were studied and the results shown in Table 4. At times, there was more than one biopsy per case for evaluation with an average of 1.5 biopsies/case. Twenty cases were diagnosed as HD and 13 as NHD. One was a low level biopsy.

The full-thickness biopsies and mucosal biopsies are described separately below.

Full-thickness biopsies

Haematoxylin and eosin and calretinin stain: A definite diagnosis of HD was made on 26 biopsies (12 cases) and of NHD on 17 biopsies (8 cases, including 5 neonates). There was 100% concordance between the two pathologists in every instance.

Table 4: Analysis of formalin fixed rectal biopsies from 33 cases of suspected HD

FORMALIN FIXED RECTAL BIOPSIES (n=56*, c=33)												
Stains Used	**FULL THICKNESS (n = 43, C= 20)**		**MUCOSAL (n-13, c-13)**									
			Adequate (n=1)		**Suspected (n=4)**		**Inadequate (n=7)**			**Low level (n=1)**		
	HD (n=26, C=12)	NHD (n= 17 C= 8)	HD	NHD	HD	NHD	HD	NHD	No diagnosis	HD	NHD	No diagnosis
H&E	26	17	0	1	4	0	0	0	7	0	0	1
Calretinin	26	17	0	1	4	0	4	3	0	0	1	0

Mucosal biopsies

H and E stain

On the basis of findings on the H and E, the 13 biopsies (13 cases) were classified as follows- NHD (1); suspect HD with hypertrophic nerves (4) or where diagnosis was not possible (8-7 inadequate and 1 low level biopsy).

Calretinin

All 13 were classified conclusively as HD (8) or NHD (5). The HD included 4 suspected on H and E and 4/7 labelled inadequate on H and E; these showed no expression. The 5 NHD cases which showed expression for calretinin included the remaining 3/7 inadequate on H and E and the single low level biopsy.

Biopsies from Ileum (3), appendix and doughnut (3):

Calretinin expression was noted in all three ileal biopsies and 1/3 appendices from suspect cases of LSHD indicating normal ganglion cell distribution. In the second appendix, ganglion cells were highlighted but no calretinin positive fibers were evident in the area of necrosis. The third appendix showed neither fibers nor ganglion cells and the AChE stain showed no ganglion cells, hence diagnostic of TCA. Interestingly, an earlier rectal biopsy of this case had showed increased AChE activity and no calretinin positive fibers, hence diagnosed as aganglionosis.

Two doughnuts from the left transverse colon assessed for circumferential innervation prior to a definitive pull through showed no increase in AChE activity, but few distinct calretinin stained fibers indicating the irregular leading ganglionated edge.

The above histologic and histochemistry findings of 74 rectal biopsies were statistically analysed [Tables 5 and 6] and found to be significant with a measure of agreement of Kappa between the two pathologists being 0.973 and between calretinin and AChE with $P < 0.001$.

Table 5: Comparison of pathologists' readings on 74 rectal samples based on calretinin.

1st Pathologist	2nd Pathologist	Number of rectal biopsies
Negative stain (HD)	Negative stain (HD)	40/74 (54.06%)
Negative stain (NHD)	Positive stain (NHD)	0/74 (0%)
Positive stain (NHD)	Negative stain (HD)	1/74 (1.36%)
Positive stain (NHD)	Positive stain (NHD)	33/74 (44.6%)

Table 6: Cross tabulation of findings obtained by the two pathologists on 74 rectal biopsies suspected of HD

Count	***First pathologist* second pathologist cross tabulation***		
	Second pathologist		***Total***
	Negative calretinin stain	***Positive calretinin stain***	
First pathologist			
Negative calretinin stain	40	0	40
Positive calretinin stain	1	33	34
Total	41	33	74

Symmetric measures				
	Value	***Asymptotic standard error[a]***	***Approximate***	
			T[b]	***Significant***
Measure of agreement Kappa	0.973	0.027	8.371	0.000
Number of valid cases	74			

[a]Not assuming the null hypothesis; [b]Using the asymptotic standard error assuming the null hypothesis; Highest consensus existed between two interpreters ($\kappa = 0.973$; $P < 0.001$)

DISCUSSION

A conclusive histopathologic diagnosis of HD on rectal biopsy is a primary requisite before planning further patient management. Conventionally, in staged procedures for HD, mucosal biopsies are preferred over full-thickness ones as they result in minimal posterior rectal fibrosis and the subsequent pull-through is technically easier.[14] However, unlike full thickness biopsies where plexuses are available for a detailed study, reporting on the minute mucosal biopsies is challenging as ganglion cells/ submucosal neural plexus may not get sampled. Hence, H and E stained mucosal biopsies must be supplemented with auxiliary stains such as AChE which demonstrate typical histochemical patterns to clinch the diagnosis of HD on the frozen biopsies. Despite minor disagreements among experts, AChE staining is a reliable diagnostic modality for HD with 100% specificity and 85% sensitivity. [15] Over the last two decades, investigators have described and compared the role of various immunohistochemical markers (e.g., PGP 9.5, S-100, NSE, GLUT-1, glial fibrillary acidic protein, Synaptophysin, MAP-5, CD 56 and Bcl 2) to AChE stain, yet none have shown any significant advantages over the latter.[6,16]

When a ganglion cell is not identified in a H and E stained rectal mucosal biopsy because of scanty submucosa, a pathologist may commit a diagnosis of HD even when hypertrophic nerve fibers are not evident. Such an error is avoided when a concurrent AChE staining is available on the frozen section. Here, a definite diagnosis can be made on a single section by demonstrating specific patterns of increased AChE activity.[11] On the other hand, if the biopsy is fixed in formalin, AChE staining cannot be performed and the absence of this robust diagnostic corroboration substantially limits the diagnostic workup in suspect HD. In this situation, one is required to examine 60-80 serial sections to confirm aganglionosis. This laborious exercise based on a negative finding is both time consuming and fraught with uncertainty. We faced this dilemma often when mucosal biopsies were received in formalin

and have felt the need for a marker that could be help identify ganglion cells/nerve patterns in formalin fixed mucosal biopsies with certainty. This study aims to evaluate the feasibility and utility of calretinin as an immune marker in the diagnosis of HD and compares it with the current standard-AChE.

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. 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.[17,18] The distinct granular network of fibers is seen in the MM and between the crypts mimicking the tree trunk mucosal pattern of staining of AChE in HD. Due to excess neuro-excitability and neuro-exhaustibility in HD, there is excess cytoplasmic calcium with loss of calretinin[19] and hence no stainable fibers with IHC. This is reflected as a "silent mucosa" in rectal biopsies of HD. Thus, negative staining with calretinin indicates HD and positive staining for calretinin indicates normal innervation. In other words, normal innervation is AChE negative and calretinin positive, while HD is AChE positive and calretinin negative.

In this comparative study, the results of calretinin IHC were comparable with rapid AChE except for one false-positive case (one pathologist) and fewer or no equivocal findings. Considering that both the pathologists have been routinely reporting AChE and H and E stained rectal biopsies for the last 10-14 years and had not reported calretinin on rectal mucosal biopsies earlier, the results are impressive. Their assessment was strictly based on the controls (full-thickness biopsies from HD) and individual positive and negative controls commercially provided with every batch of IHC staining.

We highlight certain advantages of calretinin IHC over AChE in the diagnosis of HD.

1. Frozen section facility: AChE histochemistry being a very specialized technical stain imposes a tangible burden on pathology laboratories as it is used only for diagnosis of HD. It requires technical expertise and cryostat facilities. Though our staining technique is modified to cater to a general pathology laboratory of a developing country, [11] it adds a significant work load. In contrast, Calretinin is available as one of the diagnostic markers in most laboratories with IHC facility.
2. Fresh tissue: Calretinin IHC can be performed on formalin fixed paraffin embedded mucosal biopsy that has been used earlier for conventional H and E/tissue remains after frozen H and E/AChE staining. Unlike rapid AChE, it does not require fresh and frozen tissue.
3. Adjunct to AChE staining: Since calretinin IHC can be performed on the paraffin sections of frozen remains after AChE on fresh tissue, an occasional difficulty in the interpretation with AChE can be resolved by a consensus opinion of the information obtained from all three stains — H and E, AChE and calretinin on a single fresh mucosal biopsy, provided the biopsy is handled with care.
4. Age: Calretinin stains well across all ages. Unlike the occasional problems with AChE in neonates and premature subjects, the distinct positive staining with calretinin is consistently noted in all. Some neonates with HD exhibit an “equivocal pattern” of increased AChE activity; this delicate pattern may be easily missed by the reporting pathologist unless one is aware of the entity and has an eye for it. A silent mucosa with no stainable fibers with both AChE and calretinin in a neonate is diagnostic of HD.
5. Depth of biopsy: While mucosa with MM is adequate for calretinin immunostain, except for AChE — pattern A where the superficial mucosa may be adequate for

characterization, the interpretation of other patterns (pattern B, equivocal pattern) requires the presence of submucosa in the biopsy.

6. Level of biopsy: Calretinin stained satisfactorily even on low level biopsies and provided the diagnostic information in four of our cases. Importantly, this may obviate the need for a repeat biopsy with the conventional low level biopsies when one reports only on H and E stained section.
7. Long segment disease: The increased AChE activity with typical patterns of staining in HD is restricted to the mucosa distal to the splenic flexure; it dies down proximall in the aganglionic segment in LScHD/TCA as also in the appendix in TCA. [20] In contrast, we have noted calretinin positive fibers in the entire length of colon, appendix and small intestine when they are ganglionic. This unique finding has not been highlighted in literature before and confers a decisive advantage in evaluating the colon/appendix in LScHD and TCA.
8. Calretinin is a great help to identify quadrants of abnormal innervation in doughnuts when they show no fibers and thus, prove them to be from transition zone. This is definitely advantageous over AChE where the latter fails to stain tissues when they are from proximal colon and ileum as shown in our case.
9. AChE staining requires the mixing of reagents that are toxic to human body[12] while calretinin involves minimal or no handling of toxic material.

However, there are certain disadvantages with calretenin IHC too:

1. Processing time: Since calretinin needs heat antigen retrieval for immunostaining, the formalin fixation and processing of the tissue requires a minimum of 24 hours. Unlike AChE stain, it cannot be employed on fresh tissues for intraoperative diagnosis of aganglionosis on rectal biopsy or subsequent leveling to guide a single stage pull

through. AChE staining currently requires approximately 40 min for staining a frozen section on the rectal mucosa.

2. Need for stringent control: As the negative calretinin immunostaining is a positive (diagnostic) finding in HD, it is imperative that a well-standardized and validated IHC staining protocols must be in place in order to differentiate from negative calretinin results from technical errors. This is partially overcome by running a ganglionated biopsy as a concurrent positive control in the batch as well as identifying staining of the mast cells (inbuilt positive control) in the test section.
3. Viability of biopsy: Viability of tissue is a prerequisite to study calretinin fibers. This is illustrated by the falsenegative staining of calretinin in the mucosa of ganglionic bowel noted with early ischemic necrosis. This is in contrast to AChE, which highlights fibers in aganglionosis even when mucosa shows autolytic changes at morphology.
4. Principle of staining: The aim of AChE staining is to highlight the increased parasympathetic activity seen in HD due to the absence of ganglion cells. However, calretinin staining is not based on enzyme pathology or on the primary pathogenetic mechanism, but aims at highlighting the normal calcium ion transport process in nerve processes when ganglion cells are present. It does not stain the submucosal hypertrophic nerve bundles seen in HD. However, calretinin is found to stain faintly nerve bundles in the submucosa in occasional cases of Hirschprung disease when tissue is in proximity to transition zone. [7,21]

CONCLUSION

Calretinin is a reliable single immune marker in ruling out the diagnosis of HD by positive granular staining of nerve fibers in the mucosa and submucosa of formalin fixed rectal mucosal biopsies in NHD. The improvised rapid AChEhistochemistry remains indispensable to confirm HD on fresh biopsies by demonstrating increased activity in submucosa and mucosa, thus, facilitating surgical decisions based on conclusive intra-operative diagnosis. Calretinin staining is technically less demanding and easier to interpret than AChE. Like AChE, it also reduces the necessity to study multiple serial sections for "ganglion cells" in minute rectal biopsies by routine H and E staining. In addition, it could obviate the need for repeat biopsies in low level or "superficial" rectal biopsies. Employing both stains — one positive (AChE) and one negative (calretinin) could, thus, maximize the accuracy of diagnosis in HD.

PRESENTATION

This work was presented 60th Paediatric Pathology Society (PPS) Annual Meeting (Jointly with the Society of Paediatric Pathology, SPP) at Birmingham, United Kingdom, 4th-6th September 2014. [APPENDIX- IX]

PUBLICATION

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: 369-75.

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STUDY -3

TITLE:

Role of Synaptophysin in the Intra-Operative Assessment of Quadrantic Innervation of the Proximal Doughnut in Hirschsprung Disease:

BACKGROUND:

Hirschsprung disease (HD) presents with neonatal intestinal obstruction or childhood constipation and failure to thrive. The histological diagnosis of HD rests on the absence of ganglion cells, presence of hypertrophy - hyperplasia of parasympathetic nerve bundles and increased Acetylcholine esterase (AChE) activity on frozen rectal biopsies. (1-5) The successful siting of a temporizing colostomy/ ileostomy or a definitive pull-through surgery lies on a precise identification of the proximal extent of the aganglionic colon or the distal extent of normally innervated colon (proximal doughnut). (6) Indeed, inaccurate definition of the histological transition zone (TZ) with a retained aganglionic segment of bowel or quadrantic discrepancies in the circumferential innervation in the level of bowel used for stoma / pull-through results in persistence of symptoms. (7)

Haematoxylin and Eosin (H&E) stain and AChE histochemistry have been employed on one point seromuscular biopsies and on circumferential ring bowel biopsies (doughnut) for rapid intraoperative analysis and histological leveling. As, typical AChE positive neural patterns are not well expressed in tissues proximal to the sigmoid colon, a search is on for an alternative intra operative marker to assess neural innervation status.

Synaptophysin (SY), a 38-KD membrane protein specific for the synaptic vesicles in the central and peripheral nervous system is the main constituent of AChE storage compartments,

and an important neuromuscular junction marker. (8-14) Its use as a marker for cholinergic structures and for normal neuromuscular junction has been extrapolated in this detailed prospective study on the neural innervation in 38 doughnuts of bowel from the proximal end of aganglionic segment (proximal doughnut) during histological leveling of Hirschsprung disease (HD) by estimating the difference in the density of synapses between an abnormally innervated doughnut (transitional zone/aganglionic segment) in HD and the normally innervated ganglionated doughnut.

MATERIAL AND METHODS

This prospective cross sectional study was conducted at a tertiary referral hospital for HD and allied disorders over a five-year period from 2011-2015. A suspicion of Hirschsprung disease on clinical findings and contrast enema was confirmed on a conventional rectal biopsy using improvised rapid AChE histochemistry. (2,3) After confirmation of rectal aganglionosis, proximal seromuscular point biopsies (depending on the radiologic and gross transition zones) were obtained till a normal innervation was reported; at this juncture, a doughnut of bowel was sent intraoperatively for quadrantic evaluation and final leveling by AChE histochemistry and synaptophysin IHC. This study was approved by the Institutional Ethical Board.

Doughnuts from proximal ganglionated segment were considered as normal controls. However, doughnuts from resections for habitual constipation, discontinuous aganglionosis and those where AChE histochemistry was not done were excluded.

The doughnuts sent for evaluation were received well soaked in 0.9% normal saline. They were cut into four quadrants (when big)/two (if small) and retained as one when the luminal diameter is small. They were marked and directly fresh frozen at –20ºC in the cryostat. 10 µ cryostat sections were cut from each sector and were concurrently stained with rapid H & E

for routine morphology, modified rapid acetylcholine esterase staining technique with rubeanic acid for histochemistry and immunohistochemistry for Synaptophysin marker. The frozen tissue remains were processed thereafter, for paraffin sections.

All the stained sections were labeled, coded and evaluated independently by the two pathologists (MKB, UK) in the study team. The results were statistically analyzed using SPSS 16 and p value <0.05 was considered significant. Chi Square test was used for comparative statistics and kappa agreement to document inter-observer variability, when present.

Assessment by H&E

The sections were intently studied to assess the regular distribution of ganglia in the myenteric plexus in all four quadrants (quadrantic) and for presence of hypertrophic nerve bundles.

A doughnut was considered to have normal quadrantic innervation on H&E if the ganglion cells were regularly placed in both the neural plexuses and showed no hypertrophic nerve bundles in either myenteric or submucosal plexus or both. A doughnut was considered abnormal when they exhibited irregular ganglion distribution in myenteric plexus involving one or more quadrants and/or hypertrophic nerve bundles in either myenteric or submucosal plexus.

Assessment by AChE histochemistry

The findings on H&E were ratified using AChE histochemistry for those tissues from sigmoid colon and distal to it by the demonstration of mucosal staining patterns namely A and B when AChE activity was increased and Equivocal pattern when the activity was not increased. (2,3) They are detailed as follows:

Positive pattern A: The presence of AChE positive nerve fibres in the interglandular spaces throughout the thickness of the mucosa, indicate aganglionosis and hence abnormal innervation.

Positive pattern B: The presence of AChE positive nerve fibres in the muscularis mucosa and the lowermost portion of the lamina propria below the region of the basal crypts, also indicate aganglionosis and hence abnormal innervation.

Equivocal pattern: Irregular focal presence of nerve fibres in the mucosa, muscularis mucosa or submucosa with no increase in AChE activity, indicative of normal innervation.

Negative pattern: Absence of stained nerve fibres in the mucosa with or without occasional obscure nerve twigs in the muscularis mucosa and submucosa (2) and this calls for repeat staining.

Assessment by Synaptophysin immunohistochemistry

IHC staining was carried out on frozen section by initial fixation in 10% formalin for 10 minutes and air drying for 10 minutes. They were further stained with rabbit polyclonal anti-synaptophysin antibody (clone AP10443 of Gennova Scientific) using standard protocol. (11,14) The density of synapses at 40X were assessed in the inner and outer muscularis propria, in muscularis mucosa and in muscle coat of blood vessels and scored on a three tier system of 0 to 2 (0 = absent, 1 = poor/reduced and 2 = good/abundant). Staining of neuroendocrine cells in the mucosa were considered as internal control. Counts of synapses less than half of the lowest count seen in normal control was read as abnormally diminished.

RESULTS:

Clinical Details

38 cases of HD confirmed with AChEhistochemistry on rectal biopsy (4 term neonates, 20 infants and 14 older children; 30 males and 8 females, M: F: 3.75:1) were selected for the study. They had showed features suggestive of the disease on contrast enema in 26 and inconclusive in 12 (Table 1). Two patients (a neonate and a two-year-old) presented acutely with ileal perforation and peritonitis while the rest presented with chronic constipation. 36/38 had a history of delayed passage of meconium (24-48 hrs in 22 beyond 48 hrs in 14). One child had trisomy 21 and none had culture proven sepsis.

Table 1: Patient demographic and clinical features

Characteristics	No. of Patients (n = 38)
Sex (M/F)	30/8
Neonates at biopsy	4/38
Infants at biopsy	20/38
Older children at biopsy	14/38
Delayed meconium (>24 hours)	36/38
Chronic constipation	36/38
Ileal perforation	02/38
Down's syndrome	01/38
Conclusive at radiology	26/38

Intraoperative histological and histochemical assessment

The leveling biopsies done prior to doughnut examination classified the cases as recto-sigmoid HD (RSHD) in 28, long segment colonic HD (LSHD) in 7 and total colonic aganglionosis (TCA) in 3. Identification of the transition zone was assisted by barium enema images only in recto-sigmoid HD, but was not applied for long segment colonic HD and total

colonic aganglionosis (15). The latter mandated multiple seromuscular leveling biopsies from segments of colon proximal to peritoneal reflection and the appendix / terminal ileum.

Doughnuts

The 44 doughnuts were received for quadrantic innervation studies from 38 cases of confirmed HD; 28 from 28 cases were from sigmoid and descending colon and showed normal innervation; 10 from 7 cases were from transverse colon and 6 from 3 cases were from ileum respectively and were grouped as having abnormal innervation.

Normal innervation [n= 15 control doughnuts, 28 test doughnuts (63.6%)]

H & E stain

28 test doughnuts from rectosigmoid HD which showed normal innervation were characterized by regularly spaced myenteric ganglia with distinct ganglion cells (Fig 1a) and no hypertrophic nerve bundles.

AChE histochemistry:

Equivocal staining pattern was noted with the AChE enzyme histochemistry which ratified the uniform distribution of ganglion cells in the myenteric plexus noted in H & E. Ganglion cells with their cytoplasm stained dark brown to black and staining occasional submucosal nerve fibres / nerve twigs in between the muscle fibres (Fig 1b). No staining pattern was noted in the submucosa.

Synaptophysin immunohistochemistry

Distinct saprophytic (SY) immunoreactivity was highlighted in both the layers of muscularis propria in these doughnuts. The ganglion cells showed granular cytoplasmic positivity, thus making them easily identifiable. The spindle shaped SY positive fibres identified in the muscularis propria (Fig 1c & d) were linear, elongated wavy structures located parallel to and between the muscle fibres with the mean length of 413.6µm (Fig 1d). In cross section, these are mildly irregular and bulbous with a mean diameter of 78 µm (Fig 1c). The inner circular

muscle layers were more densely innervated by the SY fibres than the longitudinal muscle layer. The synaptophysin fibres were of better intensity in cross section than longitudinal section. These fibres were also noted in the muscularis mucosa but were shorter in length. They were seen ascending into the lamina propria between the crypts reaching up to the surface epithelial lining – a pattern reminiscent of the classical AChE pattern seen in aganglionic segment of Hirschsprung disease. Distinct neuroendocrine cells located near the base and mid-portion of the crypts were highlighted by synaptophysin. The blood vessels with muscle layer showed SY positive fibres interspersed in the muscle wall. The nerve bundles which accompanied these vessels were also stained positive.

Abnormal innervation [n=16 doughnuts from 10 cases (n= 36.3%, 7 LSHD, and 3 TCA)]

H & E stain

16 doughnuts from 10 cases showed abnormal innervation characterized by absent or decrease in the number of ganglion cells and presence of hypertrophic nerve bundles in both the submucosal and myenteric plexuses to involve a variable degree of the circumference of the doughnut (Fig 2a) and were correlated with AChE and synaptophysin staining (Fig 2b to 2d). Abnormal innervation involved the entire circumference (all four quadrants) in 6 doughnuts [37.5%, 4 from LSHD and 2 from TCA], three quadrants in 1 doughnut from LSHD 6.25%, two quadrants in 3 doughnuts [18.75%, 2 from LSHD and 1 from TCA] and one quadrant in 6 doughnuts [6.25%, 3 each from LSHD and (TCA). One abnormal doughnut showed hypertrophic nerve bundles in the submucosa but regularly distributed ganglion cells in the myenteric plexus in all four quadrants (Fig. 3).

AChE histochemistry

The 16 abnormal doughnuts showed an occasional ganglion cell or none by AChE in their affected abnormal quadrants and they exhibited increased AChE activity in the submucosa by

staining the hypertrophic nerve bundles (Fig 2b) and the muscularis propria in the affected quadrants.

Synaptophysin immunohistochemistry:

Six of the 16 doughnuts with abnormal innervation characterized by aganglionosis involving all four quadrants showed no SY positive fibres in both the muscle layers of muscularis propria in the affected sectors. The lamina propria in addition, showed no SY positive fibres with occasional identifiable neuroendocrine cells in the mucosa.

The doughnuts with abnormal innervation involving one to three quarters (transitional zone) showed fewer SY positive fibers in the muscle layer (Fig 2c & d) and stained the occasional scattered ganglion cells.

The only doughnut with submucosal hypertrophic nerve bundles showed SY positive fibres in both the muscle layers but fewer SY positive fibres in the lamina propria.

The abnormal thick hypertrophic nerve bundles seen in submucosa were SY negative, unlike the serosal nerve trunks which were positive.

When the first doughnut was abnormal as in 10 cases, the operating surgeon elected to submit a second doughnut (5 cm proximal to the first) for evaluation. Only four second doughnuts from LSHD showed normal innervation whereas 3 LSHD and all 3 TCA required a third doughnut which was normally innervated in all four quadrants.

The above histologic and histochemistry findings of 15 control and 38 test doughnuts were statistically analysed and found to be significant with measure of agreement of kappa being 1.000 between the two pathologists with the p value being <0.005 (Table 2 and 3).

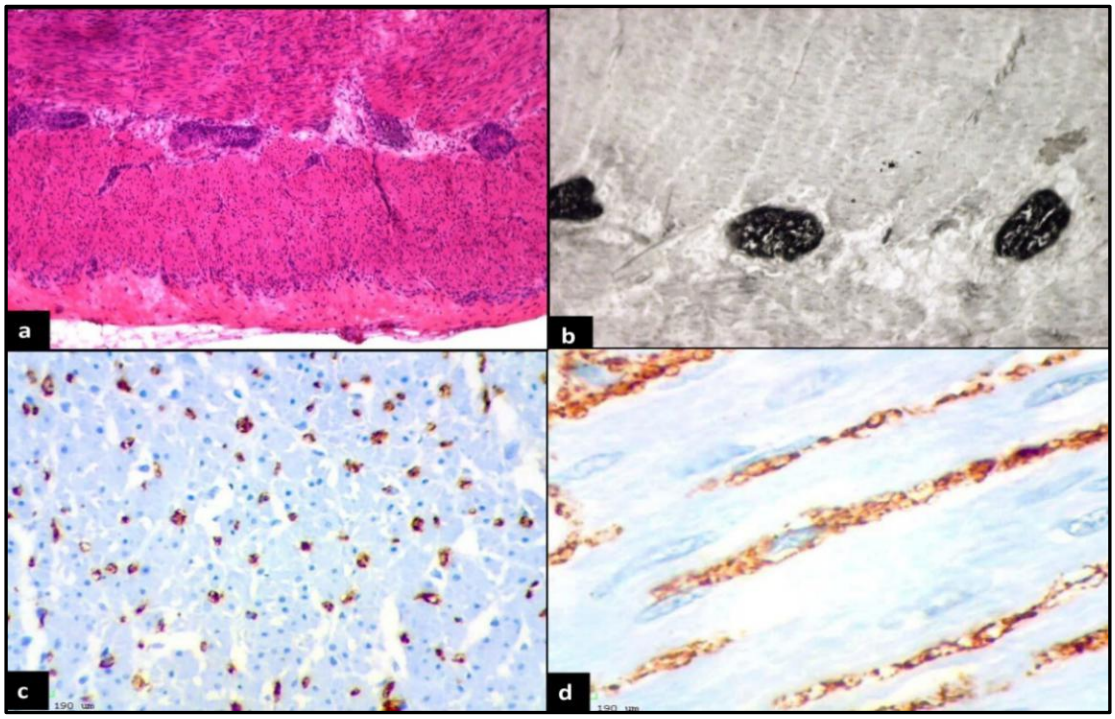

Figure 1: Normally innervated doughnut: Note **(a)** Ganglia regularly placed in the myenteric plexus. (Frozen section, H&E stain x100) highlighted by AChE stain in **(b)**. Note SY positive fibres in cross section of muscularis in **C** (x 200) and longitudinal section in **d** (X 400).

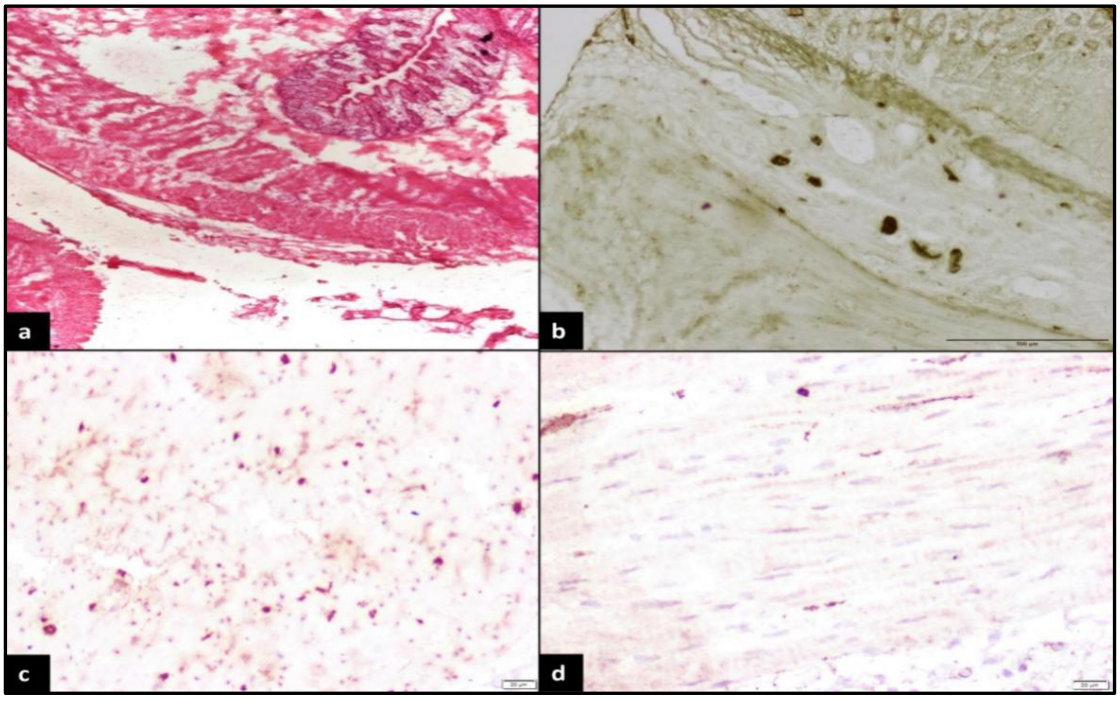

Figure 2: Abnormally innervated doughnut (a, Frozen section, H&E, x 100) and (b, AChE x 100) showing hypertrophic nerve bundles. Note decreased SY positive fibres in cross section **(c)** and in longitudinal section **(d)** of muscularis propria (IHC, Synaptophysin x 400).

Table 2: Chi-square Tests

	Value	Exact Sig. (2 sided)
McNemar Test N of Valid Test	38	1.000[a]

a. Binomial distribution used.

Table 3: Symmetric Measures

	Value	Asymp. Std Error[a]	Approx T[b]	Approx. Sig
Measure of Agreement Kappa N of Valid Cases	1.000 38	.0000	4.243	.0000

a. Not assuming the null hypothesis; b. Using the asymptotic error assuming the null hypothesis

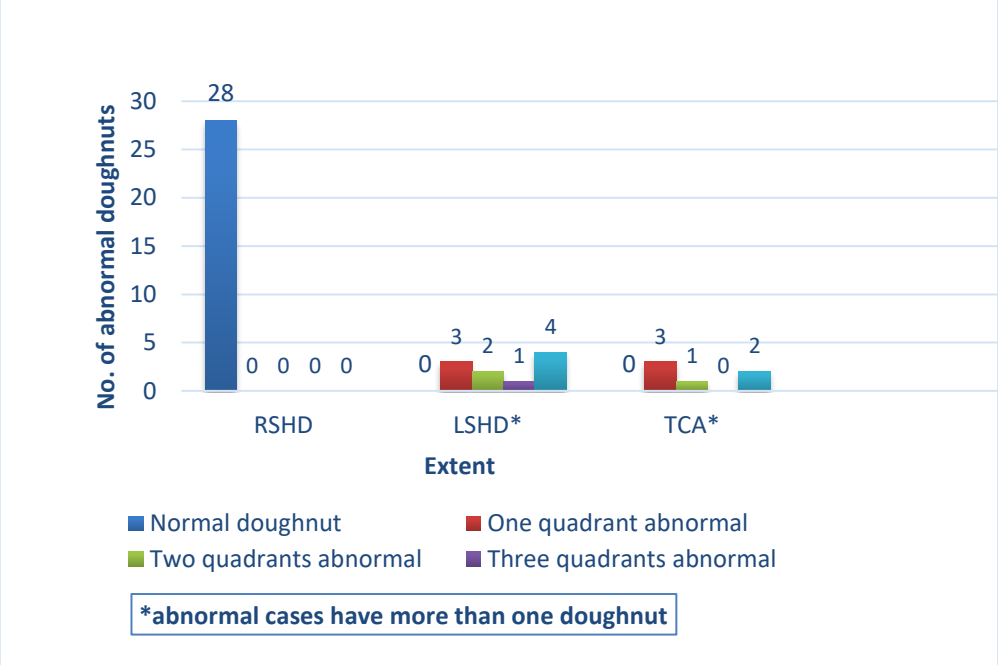

Figure 3: Bar diagram showing distribution of abnormal doughnuts in RSHD, LSHD and in TCA.

FOLLOW UP

28 of 38 cases, who had pull through with proximal doughnut showing normal innervation were followed up for a mean period of 40 months. They were asymptomatic and thrived well. They had 2-3 normal stoolings per day with formed stools and showed no persistent obstructive symptoms like abdominal distension. Initially, six of the other nine children had features of constipation which ameliorated with regular anal dilation. Since the last three months, five of these showed intermittent soiling and occasional night time incontinence and are placed on bowel management program.

One among the three children who had transitional pull through and corrected, had presented with original symptoms of abdominal distension and constipation, 3 months post- procedure. He underwent botox injection and shown amelioration of symptoms with no constipation. The other two are doing well. One child died due to unrelated cause a year later.

DISCUSSION

The assessment of ganglionic innervation by mapping seromuscular biopsies in Hirschsprung disease determines the extent of affected bowel and intraoperative biopsy is specifically geared to precisely determine the distal limit of normal innervation before a bowel stoma / pull through surgery is carried out. The minute biopsy specimens are conventionally examined using H&E staining. In certain circumstances, it is difficult to accurately identify ganglion cells in H&E sections e.g. transition zone, where it may be hypoganglionic or the ganglion cells may appear morphologically immature and hence not have classical histology. Further, a one point seromuscular (leveling) biopsy may not represent a normal innervation along the entire circumference of the doughnut due to the differential migratory nature of neural crest cells which has a leading edge. These problems are accentuated at the transition zone when one or more of the quadrants show hypertrophic nerve bundles and scattered

ganglion cells. We have reported that such transition zones need to be meticulously identified by histochemistry and managed appropriately for optimal results (16). In this study, we have investigated synaptophysin as an immunohistochemical marker for the ganglion cells (positive staining) as well as for the synapses by the demonstration of SY positive fibers. We hypothesize here that, when there is absent or decreased number of ganglion cells, synaptophysin must be proportionally lesser in number if not absent.

Synaptophysin is one of the important neuromuscular junction markers. The 38-kD membrane protein is specific for the synaptic vesicles in the central and peripheral nervous system and responsible for normal neuromuscular junction and neurotransmission. (7,8,13) Expression of synaptophysin by IHC detects intestinal ganglion cells and helps in mapping the ganglionic bowel and the synapses. Our suspicion that absence of ganglion cells is accompanied by a decrease / absence of synapses is highlighted in this study. The classical positive findings of AChE seen in Hirschsprung disease as AChE positive nerve pattern are seen in contrast to negative patterns with synaptophysin immunohistochemistry characterized by decreased number of SY positive fibers at the muscle. Thus, ganglionated segment showed intense SY immunoreactivity while AChE showed no activity and vice-versa in the aganglionated segment. Similar findings were reported by Kobayashi and Dzienis-Koronkiewicz (17) and supported by a quantitative estimation of acetylcholine esterase and synaptophysin immunoreactivity in mucosal/ submucosal and muscle layers of ganglionic and aganglionic intestinal segments in eight cases of Hirschsprung disease by Wiedenmann. (11) Using monoclonal antibody 171B5, that selectively labels synaptic vesicles, Yamataka found a similar neuronal innervation pattern in the mucosa and submucosa in aganglionic rectal specimens: only reduced numbers of unorganized synapses were seen in the lamina propria, none in the muscularis mucosae, and a few in the submucosa (18). Similarly, in both non-HD rectal and colonic specimens, many synapses arranged in neural plexuses were found

in the lamina propria, a moderate number in the muscularis mucosae and dense clusters in the submucosal plexus. Interestingly, there was a definite difference in the intensity of SY immunoreactivity between frozen doughnuts (higher intensity, greater percentage) and those fixed in formalin (lesser intensity, lesser percentage) and stained thereafter. Perhaps, SY is more easily accessible to antibodies and hence better expressed on frozen sections than on formalin fixed paraffin sections; the latter requiring heat retrieval for the same. These finding are best highlighted in this study.

CONCLUSION:

Synaptophysin immunohistochemistry as a labelling immunohistochemical method highlights the morphology of ganglion cells as well as indirectly reflect their functional status by demonstrating synapses at the level of muscle fibers on frozen sections. The pattern and intensity of this SY-positive fibre distribution in circular and longitudinal muscularis layer of colonic biopsies is an additional tool to map the ganglionic –aganglionic interface. The described pattern with synaptophysin correlates with the information gleaned from concurrent AChE staining. In ambiguous AChE cases, synaptophysin IHC staining on frozen sections is a strong valuable support in the evaluation of bowel innervations and leveling in Hirschsprung disease during intra-operative consultations.

PRESENTATION:

This paper was presented at the 61 Annual Conference of Indian Association of Pathologists and Microbiologists, 2012 held at Jamnagar, 14-16th Dec 2012. [APPENDIX- X]

PUBLICATION:

Above work is accepted for publication by The National Medical Journal of India- Manuscript No. 4979/98/2016 [APPENDIX- XI].

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STUDY -4

TITLE:

THE QUEST FOR A POSITIVE DIAGNOSTIC MARKER FOR HIRSCHSPRUNG DISEASE IN FORMALIN FIXED RECTAL BIOPSIES: A DETAILED SEVEN MARKER IHC STUDY.

BACKGROUND:

Hirschsprung disease (HD), a congenital malformation of the Enteric Nervous System (ENS) characterized by aganglionosis commonly affects the rectosigmoid region and is a leading cause of neonatal large gut obstruction and childhood constipation. HD affects 1/4500-5500 live births in the West and Japan (1,2) and 1/3000-3500 in Asiatic. (3) Though no published epidemiological data is available from India, a referral centre for HD like ours that has screened over 3000 colorectal biopsies for suspect HD over 15 years, currently diagnoses about 60 new cases annually.

Despite differences in the opinion across the Atlantic, acetylcholinesterase (AChE) histochemistry is a reliable method for the intra-operative frozen section diagnosis of HD and the subsequent levelling of aganglionosis. However, the laboratory facility with a cryostat and technical expertise for this exist in a few centres across our country. Furthermore, AChE can be performed only on fresh rectal biopsies. These logistic problems in establishing AChE histochemistry in the diagnosis of HD has simultaneously stimulated our interest in the role of diagnosis of HD by immunohistochemistry (IHC) which can be performed on formalin fixed paraffin embedded (FFPE) rectal biopsy. Hence, the aim of this study was to characterize the staining pattern of the neural plexuses with immunohistochemical neural markers such as Calretinin, GFAP, Synaptophysin, PGP 9.5, CD 56, NF and S-100 so as to

identify a positive diagnostic marker for a definite diagnosis of HD on formalin fixed rectal biopsies. We hypothesize that the neural marker should further help in differentiating the hyperplastic-hypertrophic nerve bundles characteristic of HD from the normal submucosal nerve bundles and thick extrinsic nerve bundles (in perirectal tissue serosa). The findings on full thickness biopsy, could probably be extrapolated to diagnose HD on mucosal rectal biopsies and on minute seromuscular biopsies.

MATERIAL AND METHODS:

This study was approved by the Institutional Ethical Committee (institutional IERB study no. 201/2011) and was conducted over a period of one year.

Pull through specimens bearing both aganglionic and ganglionic zones from cases of HD confirmed with AChE on fresh frozen rectal mucosal biopsies and/or formalin fixed rectal biopsy were selected for this study through convenient sampling. Tissue samples from patients with habitual constipation, or with surgical causes for constipation other than HD were excluded. Samples sent fresh or not sent in formalin were also excluded from this study.

Proximal ganglionic segments from cases of HD were considered as controls. All slides were reviewed independently by two pathologists.

Conventional Diagnosis [H&E]:

All the Hematoxylin & eosin (H&E) slides from selected cases were retrieved from the archives and studied for the best full thickness sections to include perirectal tissue/ serosa with serosal fibres. FFPE tissue blocks from ganglionic and aganglionic segments of HD were cut at 4 µm and stained with H&E. Further sections were kept aside for IHC. The sections were studied for ganglion cells and nerve fibres noting their distribution in submucosal and myenteric plexuses. The nerve fibres were studied for their distribution and staining response. Nerve fibres in the perirectal tissues were also studied in addition.

A ganglionated segment is characterized by regular distribution of ganglion cells in both the submucosal and myenteric plexuses. When there were no ganglion cells in either of the plexuses, the segment was considered aganglionic and hence Hirschsprung disease.

Immunohistochemistry (IHC):

The Novolink Max Polymer Detection System (Leica Biosystems Newcastle Ltd, Newcastle Upon Tyne NE 12 8EW, United Kingdom) was used for immunohistochemical staining on these sections.

IHC analysis was performed on 4 µm sections obtained from formalin fixed paraffin embedded blocks taken on 3- aminopropyltriethoic silane (APES) coated slides for a panel of neural markers namely Calretinin, GFAP, Synaptophysin, PGP 9.5, CD 56, NF and S-100 antibodies. The tissue sections were fixed overnight at 60^0C, deparaffinized with xylene and antigen retrieval was done by heat at 100^0C for 8 minutes in ethylene diamine tetra acetic acid (EDTA) buffer (0.1 M, pH 8.0). Endogenous peroxidase activity was neutralized by incubating the sections in peroxidase block. This was followed by application of the Protein Block to reduce non- specific binding of primary and polymer. The sections were subsequently incubated with ready to use (RTU) primary antibodies whose characteristics are shown in Table 1. Post primary antibody (Rabbit anti Mouse IgG) was then used along with the Novolink^{TM} polymer to label primary antibodies. Sections were further incubated with the substrate/ chromogen, 3, $3^{,-}$ diaminobenzidine (DAB), prepared from DAB chromogen and Novolink^{TM} DAB substrate buffer for 3 minute at room temperature and counterstained with hematoxylin. Sections were then mounted with DPX and studied for interpretation.

Table 1: Characteristics of primary antibodies used:

Sl. No.	Antibodies	Source	Clone	Titer	Antigen retrieval method
1.	Calretinin	Dako, Denmark	DAK- Calret 1	Ready to use	Heat with EDTA
2.	GFAP	Dako, Denmark	Rabbit polyclonal	Ready to use	Heat with EDTA
3.	Synaptophysin	Genova Scientific, Spain	Rabbit polyclonal	Ready to use	Heat with EDTA
4.	PGP 9.5	Novocastra Leica Biosystem, New Castle, UK	10A1	Ready to use	Heat with EDTA
5.	CD 56	Dako, Denmark	123C3	Ready to use	Heat with EDTA
6.	NF	Dako, Denmark	2F11	Ready to use	Heat with EDTA
7.	S-100	Novocastra Leica Biosystem, New Castle, UK	Rabbit polyclonal	Ready to use	Heat with EDTA

IHC evaluation:

Immunohistochemical staining with all seven markers was evaluated on sections which were coded to asses staining of neural fibres in various layer of the rectal wall and their intensity. They were interpreted independently by two pathologists blinded to the H&E diagnosis and to avoid inter-observer bias.

Staining intensity

The staining intensity for ganglion cells and nerve fibres were scored from o to 2; 0 = no staining; 1 = weak staining and 2 = strong staining. Extrinsic serosal nerve fibres, hyperplastic-hypertrophic nerve fibres in the aganglionic segment, submucosal nerve bundles in the ganglionic segment were studied in particular in addition to fibres in the lamina

propria.

The mean staining intensity for each of the seven immunohistochemical markers in each of the ganglionic, and aganglionic segments was calculated both for ganglion cells and nerve fibres.

RESULTS:

One hundred tissue blocks from 50 cases selected were studied. The age of the patients ranged from 15 days to 2 years, with a male female ratio of 3.75:1. 3.

H& E staining:

The ganglionic sections show intact mucosa with ganglion cells in the neuronal plexuses with occasional nerve bundles while the aganglionic segments showed hyperplastic-hypertrophic nerve bundles in both the neuronal plexuses with no ganglion cells (Fig, 1a and 1b). The extrinsic serosal fibres were thick and prominently noted in all sections of both ganglionic and aganglionic (Fig, 1c).

Immunohistochemical staining:

The staining pattern of ganglion cells and of nerve fibres by the various seven immunohistochemical markers and their intensities are shown below in Fig 1, Fig 2 and Table 4 and the details elaborated as below.

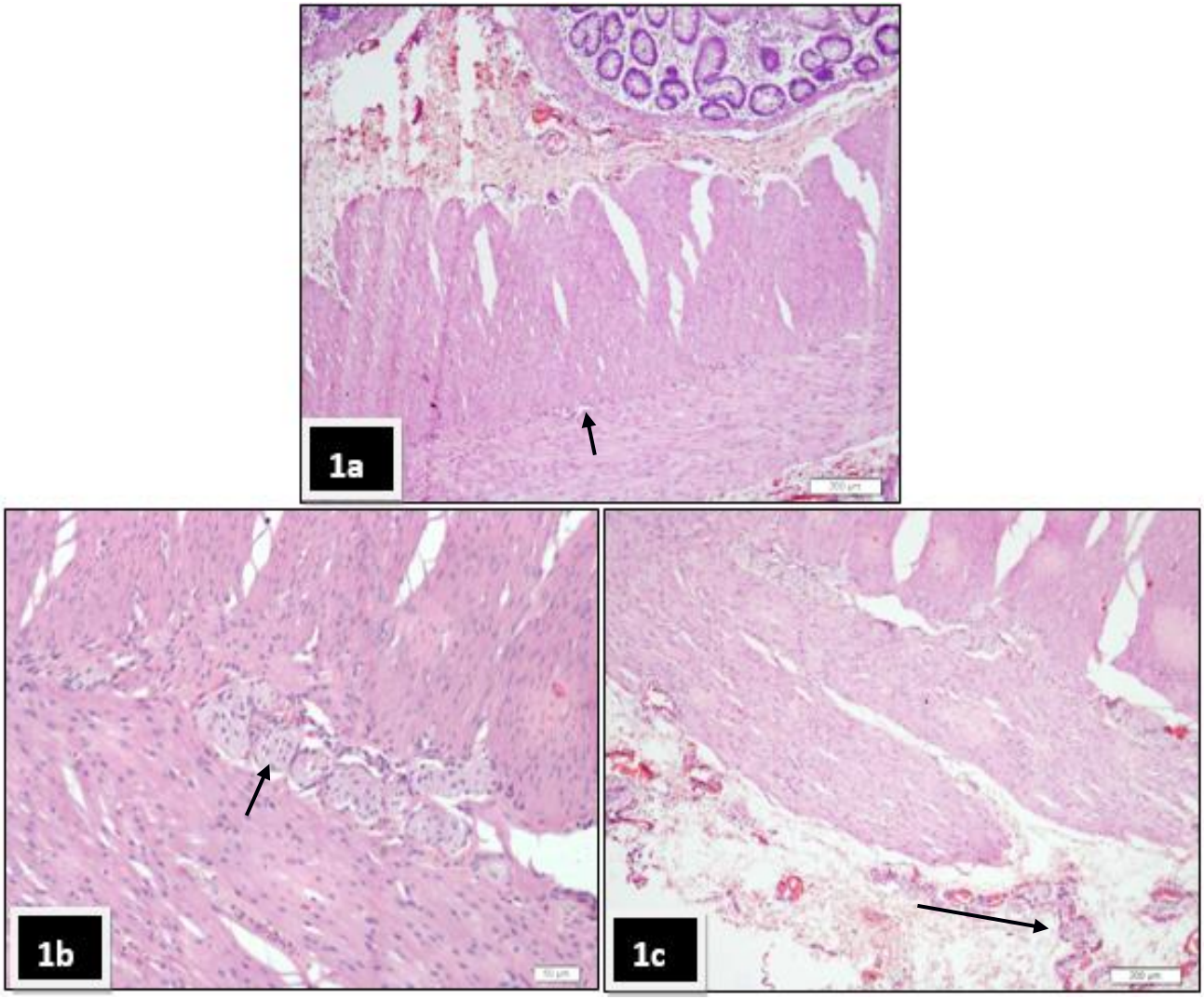

Fig. 1: H&E stain highlighting the normal myenteric plexus (arrow in black) staining the ganglion cells (arrow). (b) and (c) showing hyperplastic-hypertrophic nerve bundles in the neuronal plexuses of the aganglionic segment and serosal nerve bundles (×200) (arrows).

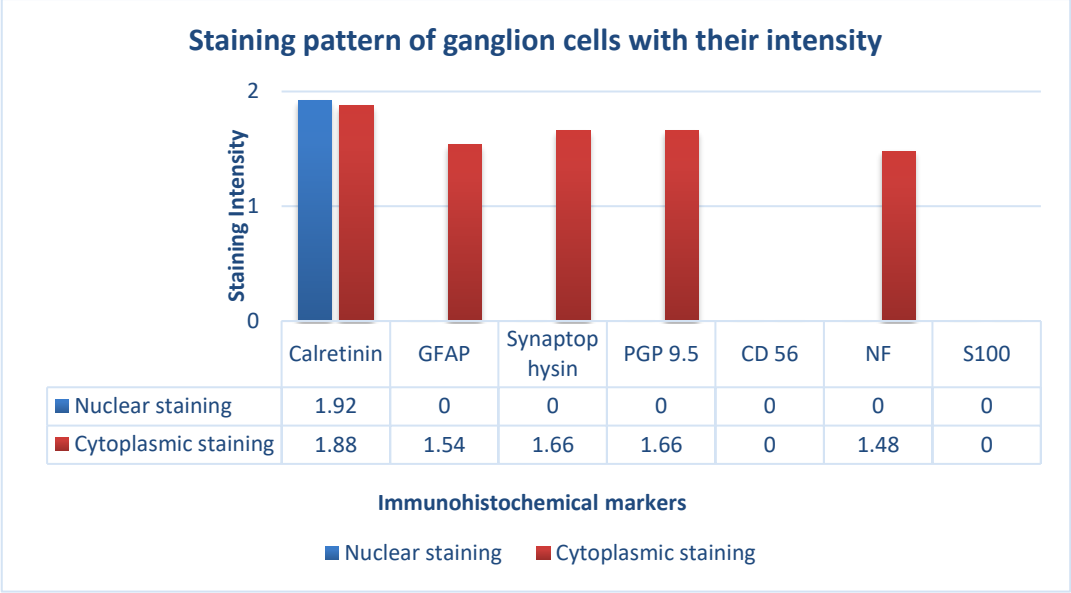

Fig 3: Bar diagram to show staining patterns of ganglion cells with their intensities.

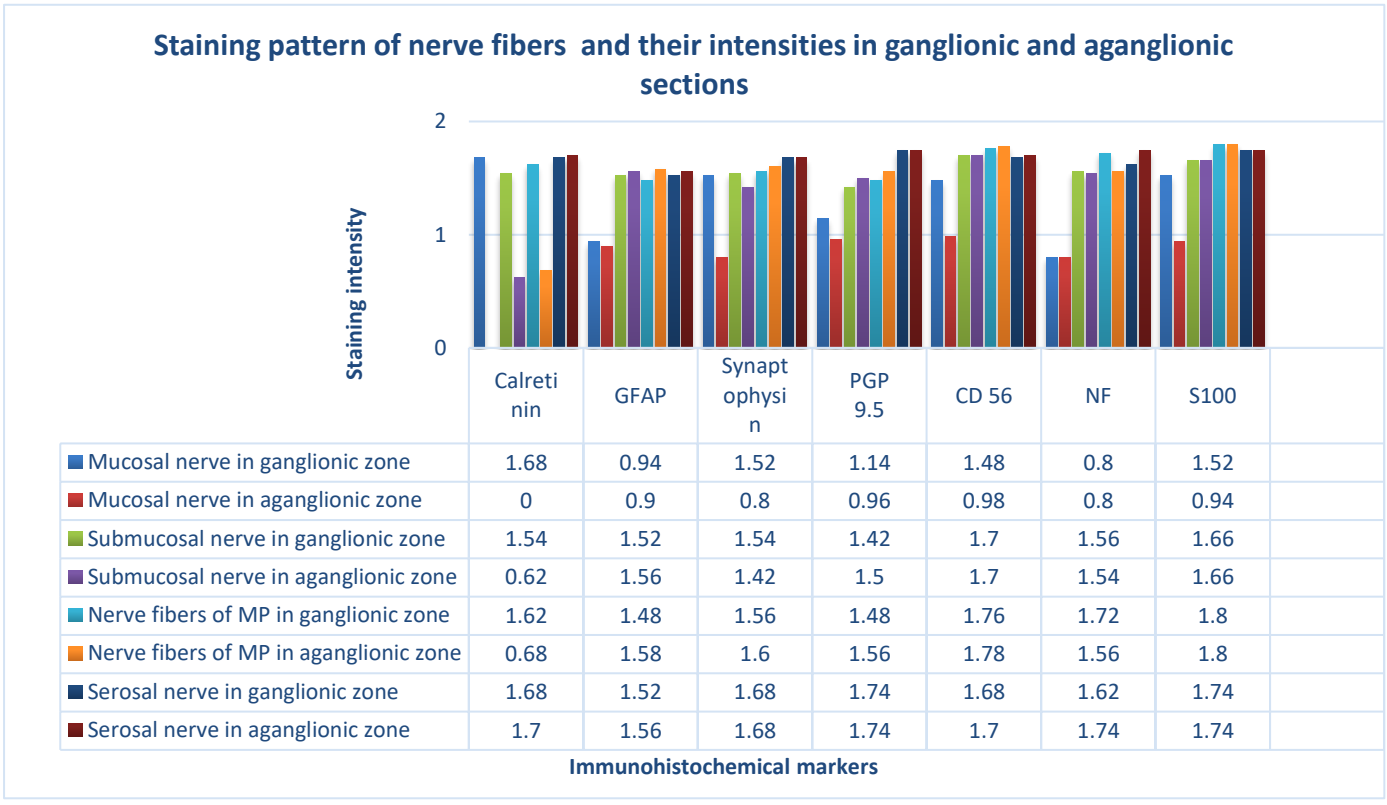

Fig 3: Bar diagram to show staining patterns of nerve fibers and their intensities at all levels in the wall in ganglionic and aganglionic sections.

Calretinin

The calretinin stained both the nucleus and cytoplasm of ganglion cell clusters (Fig 4a), the submucosal and the serosal nerves. An average staining intensity observed was 1.92 for nucleus and 1.88 for the cytoplasm (Fig 2). Distinct positive granular intrinsic fibres were seen in muscularis mucosa and lamina propria between the crypts, reaching up to the surface lining in the ganglionic segment. Positively stained mast cells acted as the positive internal control.

In aganglionic segment, calretinin failed to stain the fibres in the mucosa and hyperplastic-hypertrophic nerve bundles (Fig 4b and 4c). However, extrinsic serosal nerve fibres were highlighted.

GFAP

The GFAP stained the cytoplasm of ganglion cells (Fig 5a) with an average staining intensity of 1.54. No nuclear staining was observed. GFAP stained the nerve fibres in the submucosa and in myenteric plexus with an average staining intensity of 1.42 and 1.48 respectively but failed to show any nerve fibres in the muscularis mucosa whereas in lamina propria these nerve fibres were focally stained.

Hyperplastic-hypertrophic nerve fibres in sections from aganglionic zone were stained by GFAP (Fig 5b) with an average staining of 1.5 in submucosa and 1.56 in myenteric plexus (Fig 3). Extrinsic nerve fibres in the serosa were similarly stained (Fig 5c).

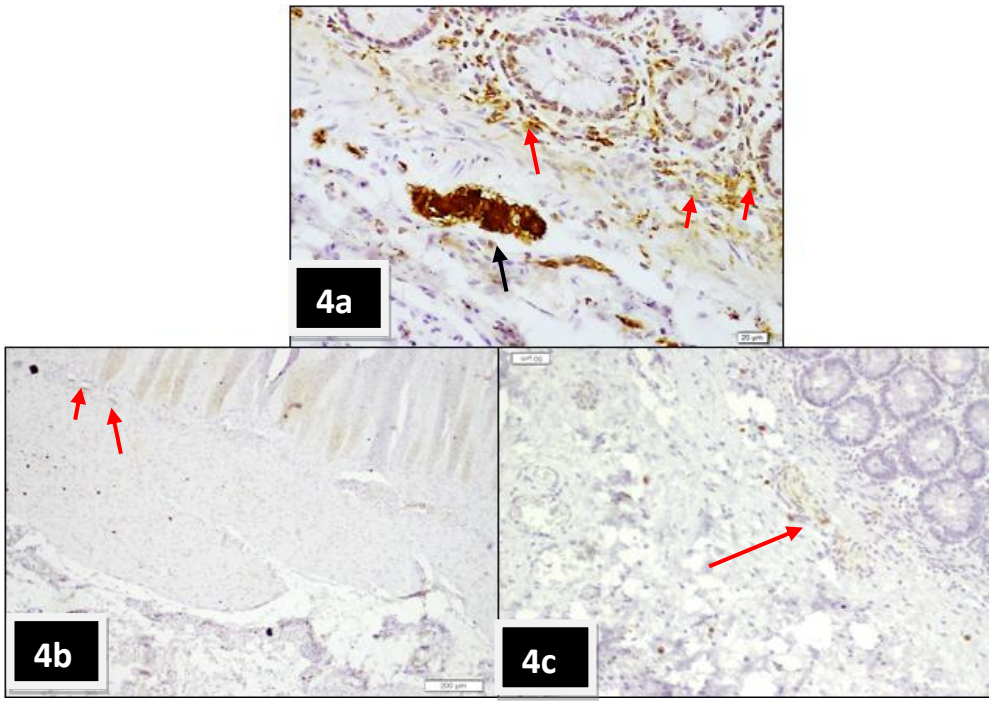

Figure 4: Calretinin immunohistochemistry highlighting the ganglion cells (arrow in black) staining both the nucleus and the cytoplasmas well as the intrinsic fibers in the mucosa (arrow in red). (b) and (c) showing unstained hyperplastic-hypertrophic nerve bundles in the neuronal plexuses of the aganglionic segment (×200) (arrows).

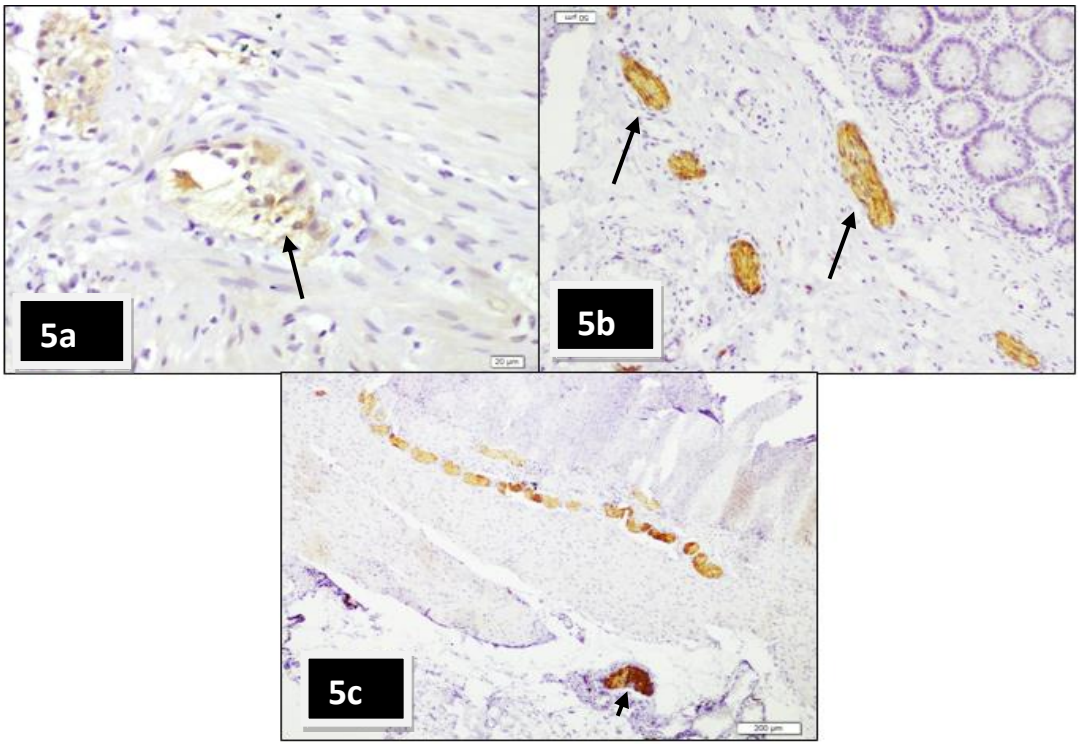

Figure 5: GFAP immunohistochemistry showing cytoplasmic staining of ganglion cells in (a). (b) showing hypertrophic nerve bundles in submucosa and myenteric plexus (arrows) as well as extrinsic nerve fibers in the serosa highlighted by the immunostain (arrow heads) (×200).

Synaptophysin (SY)

Synaptophysin stained the cytoplasm of ganglion cells (Fig 6a) but not the nucleus. Nerve fibres in the mucosa, submucosa and in myenteric plexus in ganglionic sections were also stained with an average intensity of 1.52, 1.54 and 1.56 respectively (Fig 2). In addition, it also stained neuroendocrine (NE) cells in the crypts and SY positive fibres between the muscle fibres.

In aganglionic section, SY showed immunoreactivity for hyperplastic-hypertrophic nerve fibres in submucosal and myenteric plexus with few fibres in the mucosa. An average staining intensity of nerve fibres in both the plexuses were 1.42 and 1.6 respectively (Fig 3). Extrinsic serosal nerve fibres in the serosa were also stained by synaptophysin while a dense network of synapses was observed in the muscularis of the ganglionic, the staining was poor with minimal intensity in the muscularis of the aganglionic segment.

PGP 9.5

PGP 9.5 stained the cytoplasm of the ganglion cells (Fig 7a) and nerve fibres in lamina propria, muscularis mucosa, submucosal and myenteric plexus in ganglionated sections. An average staining intensity of the cytoplasmic stain was 1.66. An average staining intensity of nerve fibres noted in mucosa, submucosal and myenteric plexus in ganglionic sections were 1.14, 1.42 and 1.48 respectively (Fig 2).

PGP 9.5 also stained the hypertrophic nerve fibres (Fig 7b) in aganglionic segment. The average staining intensity noted was 1.5 in submucosal plexus and 1.56 in myenteric plexus (Fig 3). In addition, PGP 9.5 also stained the extrinsic serosal nerve fibres in the serosa (Fig 7c). However, mucosal nerves were stained not so intense as seen in ganglionic sections.

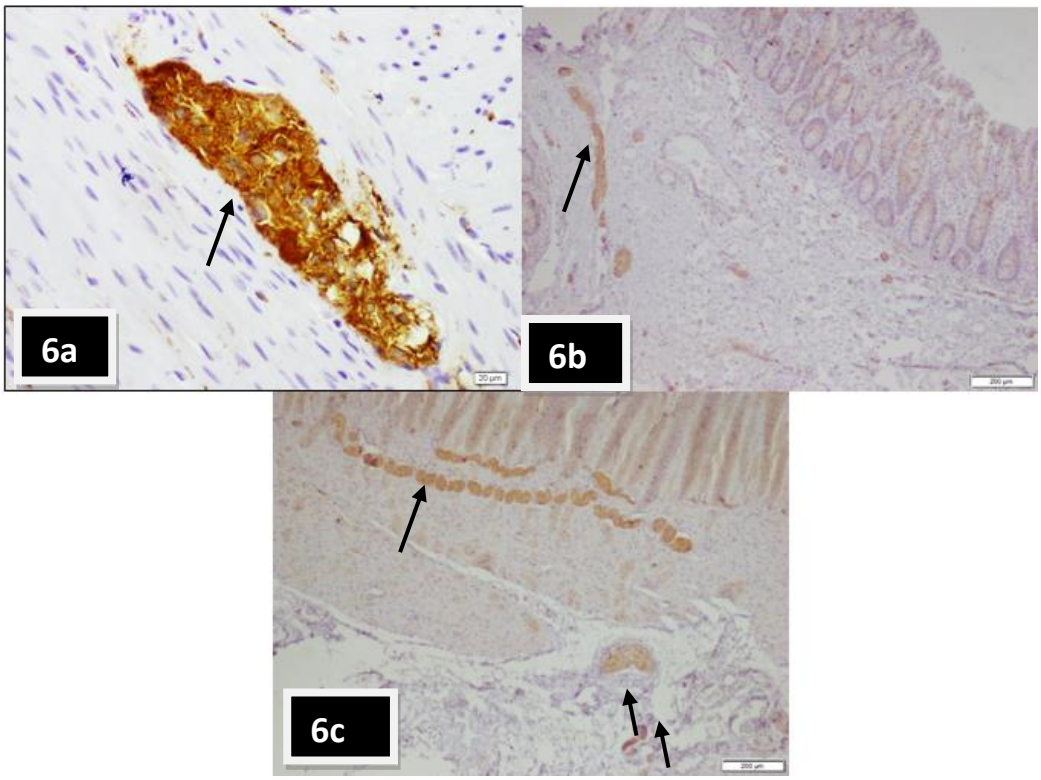

Figure 6: Synaptophysin immunohistochemistry highlighting ganglion cells in (a) (arrows) and the hypertrophic nerve bundles in aganglionic as well as the extrinsic nerve fibres (b) and (c) (arrows).

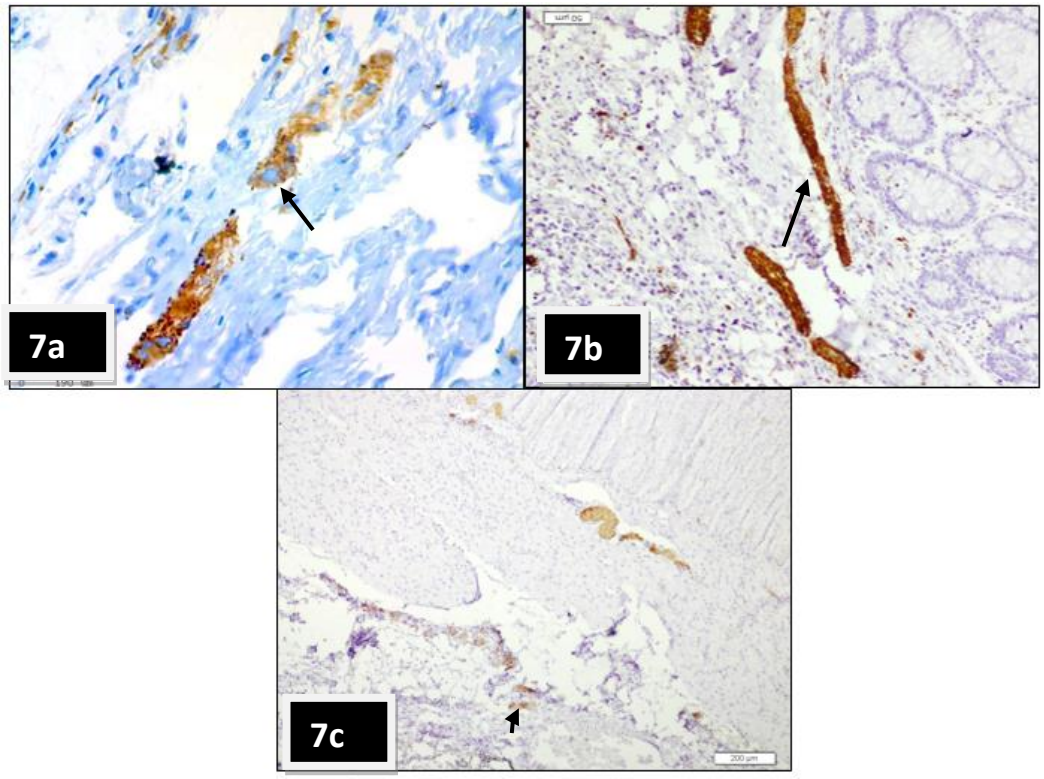

Fig 7: PGP 9.5 immunohistochemistry showing granular cytoplasmic positivity of the ganglion cells(a) (arrow) and hyperplastic-hypertrophic nerve bundles in aganglionic section(b) as well as the extrinsic nerve fibres in the serosa (c) (×200).

CD 56

CD 56 stained fibres in the muscularis mucosa and mucosa in sections from ganglionic zone. Nerve fibres in the mucosa and in myenteric plexus were also stained but did not stain ganglion cells (Fig 8a and 8b).

CD 56 showed positive staining of hyperplastic-hypertrophic nerve fibres in aganglionic sections (Fig 8c). The average staining intensity of these fibres were 1.7 in submucosal and 1.78 and in myenteric plexus (Fig 3). Extrinsic serosal nerve fibres in the serosa were similarly stained by CD56.

NF

The NF stained the cytoplasm of ganglion cells (Fig 9a) and nerve fibres in the mucosa, submucosal and in myenteric plexus.

In aganglionic sections, the NF stained hyperplastic- hypertrophic nerve fibres (Fig 9b) with an average staining intensity of 1.54 in submucosal and 1.56 in myenteric plexus (Fig 3). The NF also stained extrinsic serosal nerve fibres (Fig 9c) with the same intensity.

S-100

S-100 stains nerve fibres in the muscularis mucosa, sub mucosa and in myenteric plexus in sections from ganglionic zone with an average staining intensity of 1.52, 1.66 and 1.8 respectively but does not stain the ganglion cells.

S-100 also showed its immunoreactivity for hyperplastic-hypertrophic nerve fibres in both the plexuses in sections from aganglionic zone (Fig 10b). An average staining intensity of these nerve fibres noted were 1.66 and 1.8 respectively (Fig 3). Extrinsic serosal nerve fibres were also stained by S-100 (Fig 10c).

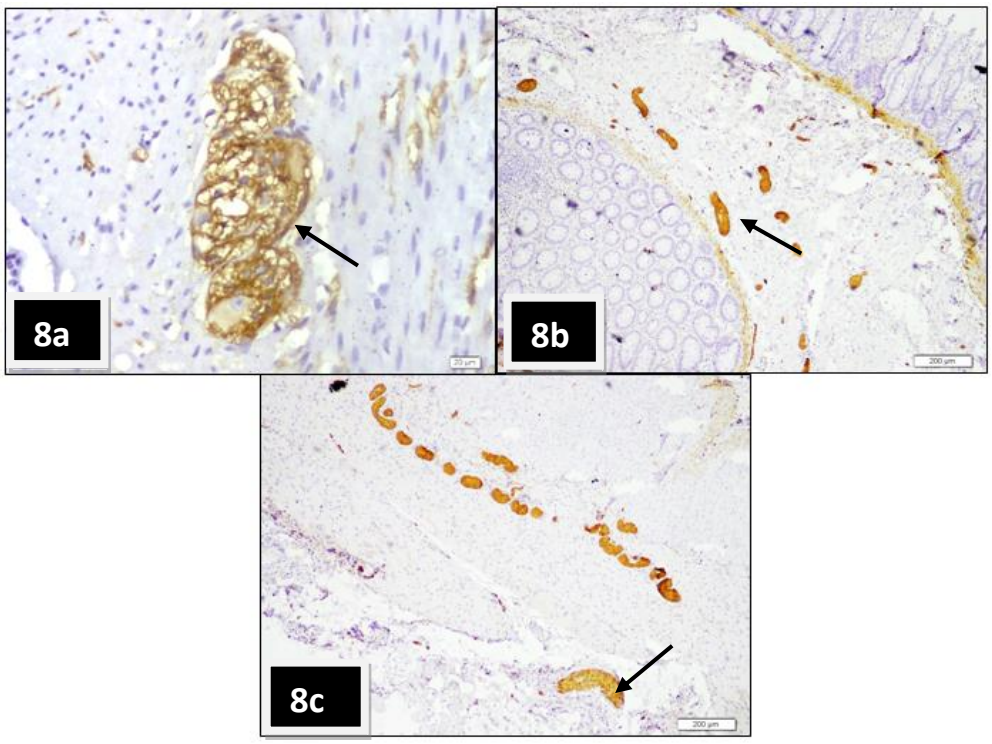

Figure 8: CD 56 immunohistochemistry showing no staining of ganglion cells as seen in (a) (arrow) but highlights hypertrophic nerve bundles in (b) and extrinsic nerve fibres in the serosa (c) (×200) (arrows).

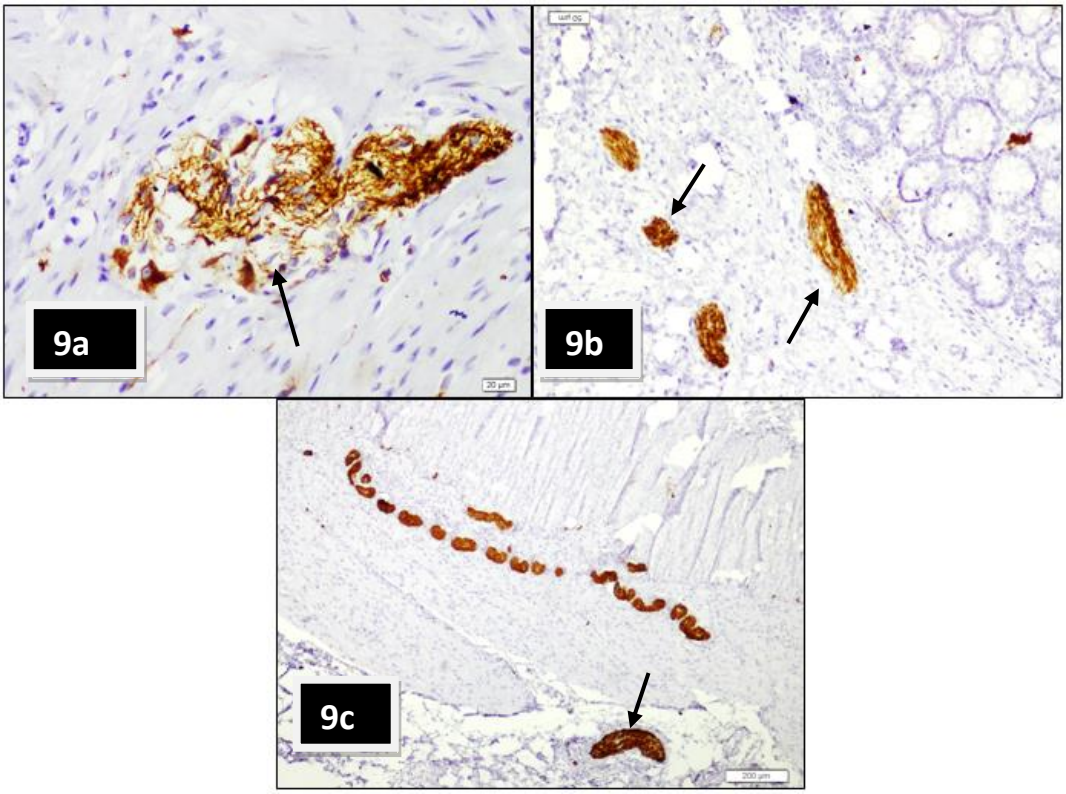

Figure 9: NF immunohistochemistry showing cytoplasmic staining of ganglion cells in (a) (arrow). (b) shows hypertrophic nerve bundles and extrinsic serosal nerve fibers in the serosa (c) (×200) (arrows).

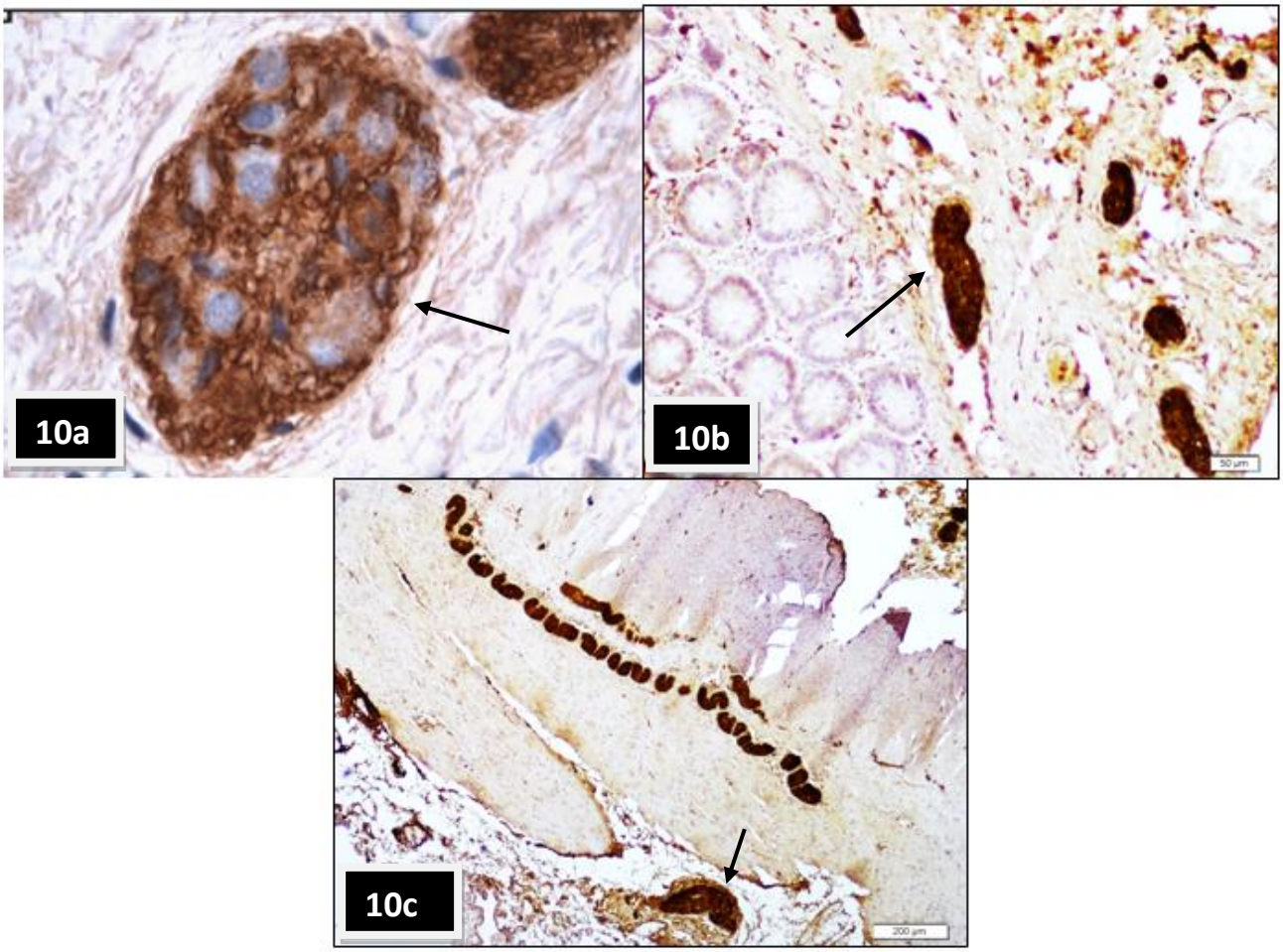

Figure 10: S-100 immunohistochemistry showing no staining for ganglion cells as seen in (a), (x 400), (b) shows hypertrophic nerve bundles in the aganglionic and the extrinsic nerve fibres in the serosa (c) (×200).

The staining patterns of ganglion cells (nuclear/cytoplasmic staining) and of the nerve fibres in ganglionic and in aganglionic zone are tabulated as shown below in Table 2 and 3.

Table 2: Staining patterns of ganglion cells and nerve fibres in ganglionic sections with the panel of neural markers

Sl. No.	IHC Neural Markers	Staining of Ganglion cells		Staining of Mucosal fibres	Staining of Normal Nerve fibres in		Staining of Serosal (Extrinsic) nerve
		+/-	N/C staining		Submucosa	Myenteric plexus	
1.	Calretinin	+	N & C	+	+	Absent	+
2.	GFAP	+	C	Seen in muscularis mucosa	+	+	+
3.	Synaptophysin	+	C	+	+	+	+
4.	PGP 9.5	+	C	+	+	+	+
5.	CD56	No staining			+	+	+
6.	NF	+	C	Seen in muscularis mucosa	+	+	+
7.	S-100	No staining		+	+	+	+

+ stained, N Nuclear, C Cytoplasmic

Table 3: Staining patterns of nerve bundles in aganglionic sections with the seven neural markers studied with IHC

Sl. No.	IHC Neural Markers	Staining of Mucosal fibres	Staining of hyperplastic-hypertrophic nerve bundles in		Staining of Serosal (Extrinsic) nerve
			Submucosa	Myenteric plexus	
1.	Calretinin	No staining	-	-	+
2.	GFAP	muscularis mucosa	+	+	+
3.	Synaptophysin	Fewer	+	+	+
4.	PGP 9.5	In muscularis mucosa	+	+	+
5.	CD56	muscularis mucosa	+	+	+
6.	NF	muscularis mucosa	+	+	+
7.	S-100	muscularis mucosa	+	+	+

Table 4: Mean values of staining intensity for calretinin, GFAP, Synaptophysin, PGP 9.5, CD 56, NF, S-100

	Calretinin	**GFAP**	**Synaptophysin**	**PGP 9.5**	**CD 56**	**NF**	**S-100**
GANGLION CELLS							
Nuclear Staining	**1.92**	0	0	0	0	0	0
Cytoplasmic staining	**1.88**	1.54	1.66	1.66	0	1.48	0
NERVE FIBRES IN GANGLIONIC SECTIONS							
Mucosal Fibres	**1.68**	0.94	1.52	1.14	1.48	0.8	1.52
Submucosal nerve	**1.54**	1.52	1.54	1.42	1.7	1.56	1.66
Nerve fibres in Myenteric plexus	**1.62**	1.48	1.56	1.48	1.76	1.72	1.8
Serosal Nerves	1.68	1.52	1.68	1.74	1.68	1.62	1.74
NERVE FIBRES IN AGANGLIONIC SECTIONS							
Mucosal Fibres	0	0.9	0.8	0.96	0.98	0.8	0.94
Submucosal nerve	**0.62**	1.56	1.42	1.5	**1.7**	1.54	**1.66**
Nerve fibres in Myenteric plexus	**0.68**	1.58	1.6	1.56	**1.78**	1.56	**1.8**
Serosal Nerves	1.70	1.56	1.68	1.74	1.7	1.74	1.74

DISCUSSION:

The present study was conducted to identify a positive immunohistochemical marker to diagnose HD on a formalin fixed rectal biopsy. H&E stains both ganglion cells as well as all types of nerve bundles in plexuses irrespective of the heterogeneity of the type of neurons, and type of nerve fibres-the normal and the abnormal. Hence, this study of seven IHC markers (Calretinin, GFAP, Synaptophysin, PGP 9.5, CD 56, NF and S-100) on full thickness formalin fixed rectal biopsy to study their expression for ganglion cells, normal, abnormal (hypertrophic) nerve fibres and extrinsic nerve fibres. This is also to explore the possibility to extrapolate the results to read mucosal biopsies on rectum for a diagnosis of HD.

The enteric neural system is divided into two nervous plexuses, namely the submucosal or Meissner's plexus and the myenteric or Auerbach's plexus. (4) Calretinin, a vitamin D-dependent calcium-binding protein, has been the subject of interest in many HD-related studies in the past decade. Barshack et al, (5) and Yadav et al, (6) proved that the calretinin antibodies stained ganglion cells in both the plexuses as well as the intrinsic fibres in the lamina propria between the crypts which could be used in the diagnosis of HD. Kapur et al (7) suggested that calretinin could be superior on formalin fixed biopsies than acetylcholinesterase staining on fresh rectal biopsies. They used calretinin staining to detect small intrinsic fibres in lamina propria and in mucosa in normally innervated tissue along with the ganglion cells. These fibres were absent in biopsies from HD as proved in our earlier study. (6) In this study, too, we found calretinin staining both the nucleus and the cytoplasm of ganglion cells in both the plexuses. Intrinsic fibres were seen in the lamina propria and in muscularis mucosa. However, in aganglionic section, the calretinin stain remained silent. None of the hyperplastic-hypertrophic nerve bundles were stained. Guinard-Samuel et al (8) made similar observations in their retrospective study. Yadav et al (6), Morris et al (9) and Gonzalo et al (10) have emphasized the role of calretinin, particularly in total aganglionosis,

inadequate superficial rectal mucosal biopsies and biopsies from premature babies. A combination of calretinin and acetylcholinesterase staining was proposed by de Arruda et al, and Yadav et al (6,11) who state that calretinin was a useful tool for ruling out HD, while acetylcholinesterase was most useful for confirming the diagnosis of HD as a positive marker. In seromuscular biopsies in which the myenteric plexus fail to show ganglion cells in view of the small biopsy size, staining with calretinin will differentiate hypertrophic nerve bundles on one side (submucosa) (which remain unstained) from extrinsic nerve bundles (serosa) which are stained positive with calretinin, thus confirming a definite diagnosis of HD in such biopsies where serosa and submucosal tissue look alike.

GFAP is an another neural marker which has been suggested in the diagnosis of HD in several studies. (12-15) Mackenzie et al. (16) compared GFAP with Neurofilaments (NF), Neuron- Specific enolase (NSE) and S-100 and found GFAP staining both normal as well as hypertrophic and hyperplastic nerve bundles with strong intensity while Schwan cells were weakly stained. They also found perikarya of ganglion cells weakly stained. Kawana et al, (17) have also compared GFAP with S-100 and found that, GFAP selectively labeled a population of multipolar, non-neuronal supportive cells bearing a morphological resemblance to astroglia and normal submucosal nerves in ganglionic segment. The nerve bundles from aganglionic segment were also highlighted. In our study, we found GFAP staining the cytoplasm of ganglion cells weakly. Nerve fibres in the mucosa, submucosa and in myenteric plexus showed mild to moderate staining intensity. However, it stained hypertrophic nerve bundles in aganglionic sections with a strong intensity.

Synaptophysin, a 38-kD membrane protein is specific for the synaptic vesicles in the central and peripheral nervous system and responsible for normal neuromuscular junction and neurotransmission. (18) It is an immunohistochemical positive marker for ganglion cells and neuromuscular junction staining nerve bundles which are both normal and hypertrophic and

extrinsic, hence does not differentiate the two as elaborated in this study as well as by Zuikova at al. (19) Interestingly, the demonstration of a rich network of SY positive synapses in this study differentiated ganglionic from aganglionic intestinal wall similar to what was observed by Dzienis-Koronkiewicz et al, (18). The pattern of SY-positive synapses distribution in circular and longitudinal colonic muscles can reflect functional disturbances of large bowel motility and could be helpful in the description of the innervation status of colonic specimens in HD patients as shown in our study. (20)

PGP 9.5, a marker of neuronal tissue which stains a few nerve fibres in the mucosa and submucosa, the muscularis mucosa, the lamina propria of formalin-fixed biopsies and the ganglion cells also stained ganglion cells intensely. In addition, PGP9.5 was found to stain nerve fibres in the myenteric and submucosal plexuses in both ganglionic and aganglionic segments. (21,22) Geramizadeh et al (23), in their study counted the number of enteric neural cells in the inner circular and outer longitudinal muscle layer separately and found that these cells were markedly decreased in aganglionic segment compared to ganglionic one. In this study, we found PGP 9.5 showing granular cytoplasmic positivity of ganglion cells, occasional fibres in lamina propria and dense staining of neuronal cells in the muscularis propria specially in outer longitudinal muscle.

Jiang et al, (21) evaluated the value of calretinin combined with S-100 and PGP 9.5 immunohistochemistry on rectal suction biopsies. They found that PGP9.5 stained the hyperplastic-hypertrophic nerve fibres intensely in HD affected tissue while in the normally innervated intestine, only granular staining of small nerve twigs were seen in the submucosa, in contrast to strong staining of ganglion cells. Similar findings were seen in this study too. supports our study.

CD 56 is a marker of neuromuscular junction. Geramizadeh et al, (23) have counted CD56 positive cells in the inner circular and outer longitudinal muscle layer in aganglionic and

ganglionic segment and found that CD 56 positive cells were significantly lower in HD. However, they have not discussed about the staining pattern of ganglion cells and nerve bundles. In our study, we have noted that CD 56 stained normal nerves in ganglionic sections and hyperplastic-hypertrophic nerve bundles in aganglionic zone. This study was also supported by Meyrat et al (24) where they have selected CD56 staining for hyperplastic-hypertrophic nerve bundles in aganglionic section to assess the colonic innervation before the pull-through procedure in HD.

Neurofilament (NF) is a cytoskeletal intermediate filament specifically and exclusively present in nerve cells, which can therefore be used as a specific marker for neurons in the enteric nervous system. Mackenzie et al, (16) discovered NF for staining pattern of nerve fibres in submucosal plexus and myenteric plexus in both ganglionic and aganglionic segment. They found that, there was strong staining of nerve fibres in both plexuses. However, nerve fibres in the lamina propria were not demonstrable except a focal staining of unmyelinated axon-like structures in only one case.

In our study, NF staining showed intense cytoplasmic immunoreactivity for ganglion cells and nerves in submucosal and myenteric plexus in ganglionic sections.

S-100 is a low molecular mass calcium-binding protein expressed in glial cells, among others. Joosten et al (25) found staining for S-100 to be useful in proving pathological innervation as a cause of unsatisfying postoperative results in the transition zone of postoperative biopsies. In a protocol in which S-100 was combined with H&E and peripherin immunostaining for the detection of ganglion cells, Holland et al (26) found it to be highly sensitive and specific. But in this study, S-100 stained all nerves irrespective of the type and hence did not differentiate between the types namely normal, hypertrophic and extrinsic nerve fibre types but showed no staining for ganglion cells which remained highlighted without stain.

The extrinsic serosal nerves were stained by all seven neural markers whereas, the hyperplastic-hypertrophic nerve fibres and intrinsic mucosal fibres were not stained by calretinin and CD 56 alone. Thus, calretinin has proved as a negative marker for the diagnosis of HD by not staining hypertrophic nerve bundles while the other six markers stained all types of nerve fibres whether normal or abnormal (hypertrophic). The ganglion cells were stained by all seven markers except S-100. Thus, S-100 and CD 56 are important as negative markers and may play a role when one has a dispute in pinpointing a cell as a ganglion cell specially in neonates. In situations, where scanty seromuscular biopsies show myenteric plexus which is not obvious and show thick nerve bundles, it's difficult to differentiate the thick nerve bundles as either belonging to serosa (normal) or submucosa (HD), calretinin immunostain with NF may be of help as a dual marker to resolve the issue. If the thick nerve bundles are stained by both calretinin and NF, they are serosal and the biopsy needs to be considered to have normal innervation. If the bundles are stained by NF alone and not by calretinin, then the biopsy indicates aganglionosis and is diagnostic of HD. But, the hunt for a positive neural marker which should stain only hypertrophic nerve bundles and not the normal neural fibres, still continues.

CONCLUSION:

In rhis detailed search for a positive marker to confirm the diagnosis of HD on formalin fixed rectal biopsy, we found none from this panel of seven IHC neural markers to be helpful to differentiate aganglionosis from ganglionosis. None of these markers specifically stained and differentiated the hyperplastic-hypertrophic nerve bundles of Hirschsprung disease from the normal nerve bundles and extrinsic serosal nerves. However, a few in this study have proved to be negative markers especially calretinin.

Calretinin has proved to be a negative marker for the diagnosis of HD by not staining

hyperplastic-hypertrophic nerve bundles. Likewise, S-100 and CD 56 also do not stain ganglion cells. The latter may play a role when one has a dispute in pinpointing a cell as a ganglion cell specially in neonates. Calretinin with NF as a dual marker will be a great help in minute seromuscular biopsies; NF by its positivity identifies the hypertrophic nerve bundles while the same nerves remain unstained with calretinin thus proving them as abnormal nerve bundles of Hirschsprung disease.

Thus, the quest for a positive diagnostic marker in the diagnosis of HD continues with the proposal for a next panel, probably, neural growth factor receptors (NGFR)/Glut 1 by their principle of highlighting the perineurium on formalin fixed rectal biopsies.

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STUDY -5

TITLE:

DIVERSITY OF RET PROTO-ONCOGENE MUTATION IN AN INDIAN SUB POPULATION OF HIRSCHSPRUNG DISEASE: A PILOT STUDY.

BACKGROUND OF THE STUDY

Hirschsprung disease (HD) is a congenital disorder of enteric nervous system (ENS) characterized by complete absence of ganglion cells in the myenteric and submucosal plexus of intestine. This developmental defect arises from a failure of the cranio-caudal migration, proliferation, differentiation or colonization of precursor of enteric neural crest cells (ENCCs) in the intestine. (1) The precursor of the ENS originate from neural crest (NC) cells, which migrate from the vagal and sacral portions of the NC, populate the primitive gut and differentiate into neural ganglions and glial cells lining the gastrointestinal walls. (2,3)

The length of the aganglionic segment has been used to classify HD into rectosigmoid Hirschsprung disease (RSHD), long-segment Hirschsprung disease (LSHD), and total-colon aganglionosis (TCA). Its incidence is approximately 1/5,000 human live births, and has a male preponderance of 4:1. (4)

Several genes have been reported to be responsible for HD in humans including the RET gene (5), the GFRa1 gene (6), the EDNRB gene, the EDN4 gene (7) and the SOX10 gene. (8) The RET proto-oncogene is considered as the major candidate gene for causing HD and account for ~ 50% of the familial and 7- 35% of the sporadic forms of HD. (9)

The RET gene encodes the RET receptor tyrosine kinase which is expressed in neural crest-derived cells and the kidneys. RET protein is required for normal ENS development. (10)

GFRa is necessary for activation of RET protein in the presence of their ligands such as glial cell line-derived neurotrophic factor (GDNF), neurturin, artemin, and persephin. (11) Activating RET protein triggers the proliferation, differentiation, and survival of ENS cells. (12,13) When there are mutations in the RET locus, there is abnormal development of the ENS, and mice lacking RET fail to develop ENS and exhibit total intestinal aganglionosis. (14,15)

Mutations of RET gene have also been associated with multiple endocrine neoplasia type 2 (MEN 2) along with HD. (16) Mutations in six exons of RET viz, RET 10, 11, 13, 14, 15 and RET 16 exons are considered as risk exons in families with medullary thyroid carcinoma in Czech Republic. (17) Similar results were also seen in medullary thyroid carcinoma in Japanese population. (18) Mutations in different exons including exons 10, 11, 13, 14, 15 and 16 of RET gene were also seen in patients with HD. (19-21) Mutations reported in these six exons of RET gene in HD are C609W, E921X, S690P, S767R, R873Q, F893L, K907E, E921K, Cys 620 Arg, Cys 618 ser, Cys 609 Try, Cys 620 Trp, Gly 691 Ser, L769L, S899S, T654A, T706A, P679P, S836S, S904S. (4,22-28)

However, there is no published report for mutations in RET gene associated with Hirschsprung disease in the Indian population. The search for possible novel mutations in 10 to 16 exons of RET gene in HD in the Indian population would probably also show association with length of aganglionosis and help to characterize such patients further. Hence, the aim of this study is to determine the alterations in six exons of RET gene in Indian patients with Hirschsprung disease.

Materials and Methods:

This study was approved by an Institutional Ethical Committee and informed consent was obtained from all subjects for the genetic analysis.

Clinicopathologic details:

15 cases of confirmed HD with H&E and AChE on rectal biopsies (5 term neonates, 2 infants and 8 older children; 13 males and 2 female) were selected for the study along with 15 from age matched control samples who were suspected to have Hirschsprung disease but subsequently diagnosed otherwise.

Test samples:

Their presenting data and demographic data are tabulated below in Table 1. Two patients (a neonate and four-year-old) presented with ileal perforation while the rest presented with chronic constipation. All 15 had a history of delayed passage of meconium (24- 48 hours). One child was a case of Shah Wardenberg syndrome and none had culture proven sepsis.

Table 1: Patients demographic and clinical features

Characteristics	No. of patients (n = 15)
Sex (M/F)	13/2
Neonates at biopsy	5/15
Infants at biopsy	2/15
Older children at biopsy	8/15
Delayed passage of meconium (> 24 hours)	15/15
Chronic constipation	13/15
Ileal perforation	2/15
Shahwardenberg Syndrome	01/15
Conclusive at radiology	12/15

Collection of Blood Sample:

EDTA blood samples from 15 confirmed cases of Hirschsprung disease (HD) from the Indian population using simple random sampling technique were collected; 5 each from rectosigmoid HD, 5 from long segment HD, 5 from total colonic aganglionosis (TCA). They were studied along with 15 EDTA blood samples taken as controls from suspected cases of HD which were diagnosed as non-HD.

Extraction and quantification of DNA:

Whole blood samples collected in EDTA for the study were processed for extraction of DNA. DNA of each sample was extracted using QIAamp genomic DNA kit from QIAGEN.

Principle and procedure:

QIAamp DNA Blood Mini Kits are designed for rapid purification of an average of 6 µg of total DNA from 200 µl of whole human blood, and up to 50 µg of DNA from 200 µl of buffy coat. The procedure is suitable for use with whole blood treated with citrate, heparin, or EDTA, and buffy coat. Samples may be either fresh or frozen.

Lysis with QIAGEN Protease or proteinase K:

Proteinase K is the optimal enzyme for use with the lysis buffer. It is completely free of DNase and RNase activity. The activity of the proteinase K solution is 600 mAU/ml solution (or 40 mAU/mg protein). This activity provides optimal results.

Purification on QIAamp Mini spin columns:

The QIAamp DNA purification procedure comprises 4 steps and is carried out using QIAamp Mini spin columns in a standard microcentrifuge, or on a vacuum manifold. The procedures are designed to ensure that there is no sample-to-sample cross-contamination and allow safe handling of potentially infectious samples.

Adsorption to the QIAamp membrane:

The lysate buffering conditions are adjusted to allow optimal binding of the DNA to the QIAamp membrane before the sample is loaded onto the QIAamp Mini spin column. DNA is adsorbed onto the QIAamp silica membrane during a brief centrifugation step. Salt and pH conditions in the lysate ensure that protein and other contaminants, which can inhibit PCR and other downstream enzymatic reactions, are not retained on the QIAamp membrane.

Removal of residual contaminants:

DNA bound to the QIAamp membrane is washed in 2 centrifugation steps. The use of 2 different wash buffers, Buffer AW1 and Buffer AW2, significantly improves the purity of the eluted DNA. Wash conditions ensure complete removal of any residual contaminants without affecting DNA binding.

Elution of pure nucleic acids:

Purified DNA is eluted from the QIAamp Mini spin column in a concentrated form in either Buffer AE or water. Elution buffer should be equilibrated to room temperature (15–25°C) before it is applied to the column. Yields will be increased if the QIAamp Mini spin column is incubated with the elution buffer at room temperature for 5 minutes before centrifugation. The eluted genomic DNA is up to 50 kb in length (predominantly 20–30 kb) and is suitable for direct use in PCR.

If the purified DNA is to be stored, elution in Buffer AE (10 mM Tris·Cl; 0.5 mM EDTA; pH 9.0) and storage at –20°C is recommended.

Equipment and Reagents Used:

- Ethanol (96–100%)
- 1.5 ml microcentrifuge tubes
- Pipet tips with aerosol barrier

- Microcentrifuge (with rotor for 2 ml tubes)
- Vortex
- Water bath or heating block at 56°C
- Phosphate-buffered saline (PBS) may be required for some samples
- Buffers; AL, AW1 & AW2

Quantification Using Qubit 2.0 Fluorometer:

Extracted DNA were quantified using Qubit 2.0 Flurometer and the results were documented. Two assay tubes for standards, S1 and S2, and one tube each for each sample to be quantified was labeled and set up. The Qubit working solution was prepared by diluting the Qubit reagent, 1:200, in the Qubit buffer (provided in the kit). A 200 µl of working solution for each standard and sample was then prepared as follows (table 2).

Table 2: Reaction mix for Qubit Assay

	Standard Assay Tubes (S1 and S2)	Sample Assay Tubes
Volume of working solution	190µl	199µl
Volume of standard	10µl	-
Volume of sample	-	1µl
Total volume	200µl	200µl

The tubes were then vortexed for 2-3 seconds and incubated at room temperature for 2 minutes. The S1 and S2 tubes were then inserted into the Qubit fluorometer and the readings were noted as standards. Following this each tube with the sample was then quantified and the values noted (Table 3).

The quantified DNA was stored at -20°C for further use.

Table 3: Quantified values of DNA samples allotted for study.

Sl. No.	Sample Code No.	DNA Quantified Value (ng/µL)
C1	MB 07	114
C2	MB 10	175
C3	MB 12	93.8
C4	MB 13	81
C5	MB 57	55.0
C6	MB 08	140
C7	MB 14	106
C8	MB 16	125
C9	MB 29	316
C10	MB 18	398
C11	MB 35	121
C12	MB 21	236
C13	MB 28	210
C14	MB 32	52.2
C15	MB 30	336
T1	MB 07	130
T2	MB 08	148
T3	MB 19	117
T4	MB 22	199
T5	MB 37	106
T6	MB 42	125
T7	MB 50	316
T8	MB 55	48.0
T9	MB 58	121
T10	MB 60	98
T11	MB 67	236
T12	MB 69	210
T13	MB 70	52.2
T14	MB 72	336
T15	MB 100	111

Polymerase Chain Reaction (PCR) for the amplification of the gene of interest:

The Polymerase Chain Reaction is a technique used to amplify a selected region of interest of the extracted DNA; either an entire gene or a part of a gene. It involves three main steps:

Denaturation, Annealing & Extension. First, the extracted DNA is denatured, converting the double stranded DNA molecules to single strands. The primers are then annealed to the complementary regions of the single stranded molecules. In the third step, they are extended by the action of the DNA polymerase. All these steps are temperature sensitive and the common choice of temperatures for these three steps are 94^{o}C, 60^{o}C and 70^{o}C respectively. Good primer design is essential for successful reactions.

Instruments and Reagents used:

Reagents/ chemicals used

1. **For PCR:**
    - ❖ PCR Master Mix: 10x buffer, $MgcL_2$, dNTPs, Taq Polymerase; Invitrogen Life Technologies.
    - ❖ Primers: Self designed; out sourced to Sigma Aldrich for synthesis
    - ❖ DNA: Extracted, quantified & stored DNA used.
    - ❖ Nuclease free water

2. **For Gel electrophoresis:**
    - ❖ Agarose Powder 9539-500G; Sigma
    - ❖ 50X TAE Buffer; Diluted to 1X concentration
    - ❖ Sybr Safe; Ready to use; Life Technologies
    - ❖ Gel loading dye; fermentas

3. **For post PCR purification:**
    - ❖ EXO SAP IT; Bio nova supplies; Catalogue no: 78250

Instruments used

1. Centrifuge; Thermoscientific; Model: MicroCL 21
2. Vortex; Remi

3. Mastercycler gradient; Eppendorf
4. For Agarose gel electrophoresis: Power pack, casting tray, gel tank Bio-rad
5. Gel documentation system; Chemidoc XRS + imaging system, Bio Rad.

The entire process involved for obtaining the amplified products of the region of interest can be divided into 3 parts:

1. Primer designing and synthesis
2. Standardization of the primers with the extracted DNA
3. Electrophoresis & Gel documentation of the amplified DNA/ PCR product

1. **Primer Designing:**

   A primer is a segment or a short piece of DNA complementary to a given DNA sequence that acts as a point at which replication can proceed, in the presence of an enzyme like DNA polymerase.

   Primers (forward and reverse) for six exons (10, 11, 13, 14, 15 and 16) for RET gene were designed using NCBI (National Center for Biotechnology Information) tool from human RET whole genome sequence and the same is shown in Table 4 along with the sizes of their products. Once the primers were designed, they were outsourced for synthesis. The synthesized primers were then stored at -20°C for further use.

Table 4: Primers of exons 10, 11, 13, 14, 15 and 16 with their PCR product size.

Exons	PCR amplification	Size of PCR Product
10	GGGCCTATGCTTGCGACACCA	373 bp
	CCAGAGGGAGGGAGGGAAGTTT	
11	GGTCTAGGAGGGGGCAGTAAATGG	561 bp
	CAGCGTTGGCAGCCCCTCACAG	
13	AGAAGCCTCAAGCAGCATCGTC	346 bp
	AGGAGCAGTAGGGAAAGGGAGAAA	
14	ACGAGCAGCAGGAGGCAGAGA	548 bp
	GAGTGTGGCATGGTGGGGGAGTGG	
15	CCCCCGGCCCAGGTCTC	354 bp
	GCTCCACTAATCTTCGGTATCTTT	
16	GGCCTTCTCCTTTACCCCTCCTT	336 bp
	CAGCCATTTGCCTCACGAACCC	

2. Optimization of PCR (Polymerase Chain Reaction) condition for amplification and sequencing:

Ideally the annealing temperature of the primers should be 5°C lower than the melting temperature (Tm; temperature at which one half of the DNA duplex will dissociate to become single stranded and indicates the duplex stability). Primers with melting temperatures in the range of 58-64^{o}C generally produce the best results. Primers with melting temperatures above 65^{o}C have a tendency for secondary annealing. Based on this, the annealing temperature is calculated as follows: 4(G+C) + 2(A+T) – 5, where G,C,A & T are the respective number of bases seen in the primer sequence.

To optimize the PCR condition for amplification and sequencing, DNA of a control samples were treated with following reagents of standardized volume to make a PCR master mix

Nuclease free H_2O- 12.5 µL

10 x buffer - 02.0 µL

$MgcL_2$ - 01.2 µL

dNTPs - 01.0 µL

Forward Primer - 01.0 µL

Reverse Primer - 01.0 µL

DNA template - 01.0 µL

Taq Polymerase - 0.3 µL.

The PCR master mix was set to run for 35 cycles PCR under condition of:

- initial denaturation at 95^0C for 5 minutes
- denaturation at 94^0C for 30 seconds
- Annealing at 55^0C for 1 minute
- Extension at 72^0C for 1 minute
- Final extension at 72^0C for 1 minute.

The PCR was continuously repeated with different annealing temperatures of 55^0C, 62^0C and temperatures ranging from 60^0C to 67^0C using gradient PCR program until entire six exons got optimized. The reactions were carried out in a mastercycler gradient.

Optimization of annealing temperatures was interpreted seeing bands of PCR product on 1- 1.2% agarose gel using BIORAD Molecular Image Chemi DOC XRST Imaging System.

3. Gel Electrophoresis to check the DNA bands:

Ultra-pure agarose (1 Gm) from Invitrogen was dissolved by heating it in 100 mL of 1x TAE buffer. 3 µL of SYBR safe DNA gel stain from Invitrogen life technologies (catalog no. s33102) was mixed well with this solution to visualize DNA bands in

agarose. The solution was then poured to the gel tray fitted with its comb for wells. After solidification of agarose, 4 µL of PCR product is mixed well with 1 µL of DNA loading dye from fermentas (Product No. 11541575) to track the progression of gel electrophoresis and the mixture was loaded in wells of agarose gel against 3 µL of DNA ladder and control sample. The tray with gel and samples loaded in its wells was then transferred to the gel electrophoresis unit loaded with about 800 ml of 1X TAE buffer. The gel electrophoresis unit was then run at 100 volts for about an hour and the bands obtained at the end of the period were read using BIORAD Molecular Image Chemi DOC XRST Imaging System.

Cleaning of PCR Products:

After reading the bands, the remaining products were cleaned up by enzyme based method using Exo-SAP reagents. When PCR amplification is complete, any unconsumed dNTPs and primers remaining in the PCR product mixture will interfere with these methods. Exo- SAP utilizes two hydrolytic enzymes-

a) **Exonuclease I:** The exonuclease I degrades residual single stranded primers and any extraneous single stranded DNA product by the PCR.
b) **Shrimp Alkaline Phosphatase:** The Shrimp Alkaline Phosphatase hydrolyses remaining dNTPs from the PCR mixture which would interfere with the sequencing reaction.

Procedure to clean PCR product:

- For every 3.5 µL of PCR product, add 1 µL of Exo-SAP solution.
- Incubate the mixture at 37^0C for 15 minutes.

Incubate the mixture again at 80^0C for 15 minutes.

4. Sequencing of the amplified DNA (PCR product):

Sequencing is the process of determining the order/position of the 4 bases in a DNA molecule. This helps in the identification of variations in the DNA. After cleaning the PCR products, the PCR products along with their primers of respective exons, were sequenced by Sanger sequencing method using automated ABI 3730 DNA analyzer at Centre for Cellular and Molecular Platforms (C-CAMP), a sister concern of National Center for Biological Science (NCBS).

Principle:

In the enzymatic **Sanger dideoxy procedure** the sequence is determined by making a copy of the single-stranded DNA, using the enzyme DNA polymerase. DNA polymerase uses deoxyribonucleoside triphosphates (dNTPs) as substrates and adds them to a primer. The primer is hydrogen bonded to the 3' end of the DNA to be sequenced. The DNA with the primer is divided into four separate reaction mixtures. Each reaction mixture contains all four dNTPs and in addition, one of the four dideoxy analogs (dideoxyribonucleoside triphosphates; ddNTPs) of the deoxyribonucleoside triphosphates. Because in the dideoxy sugar the 3'-hydroxyl has been replaced by hydrogen, continued extension of the chain cannot occur. The dideoxy analog thus acts as specific chain-termination reagent. Fragments of variable length are obtained depending on the ddNTP in the mixture. The formed nucleic acid fragments are visualizing by using either a labelled (radioactive or fluorescent) primer or dNTPs (Fig 1).

The results of sequencing seen as electropherogram/chromatogram format were analyzed using Finch TV. Chromatogram is the visual representation of a DNA sample produced by a sequencing machine. Chromatograms of each exon were

analyzed and compared with the gene bank using ClustalW2 alignment tool to read mutations.

5. **Protein Structure Analysis using bioinformatics:**

   A change in the DNA sequence can result in a change in the amino acid sequence, which in turn may change the structure of the protein for which the gene codes i.e. affects the function of the gene. The DNA sequences of the patients and controls that were obtained were converted into amino acid sequences using an online conversion tool, EX-PASY. The amino acid sequences were latter compared with that of the standard sequences of the exons 10, 11, 13, 14, 15 and 16 to detect any changes. Once a change was detected in the amino acid sequence, the secondary and tertiary structure of the protein was predicted using the Phyre 2 server.

RESULTS:

1. **<u>Primers designing and optimization</u>:**

   To optimize the primers, the reactions for all the exons were performed at an annealing temperature of 55^0C. Analysis of the PCR products suggested non-specific bands as shown in figure 2 for all the six exons. Following this a gradient PCR was performed to obtain a suitable annealing temperature. The temperatures range for gradient PCR was set between 60 to 67^0C. Analysis of the PCR products showed the clean bands as shown in figure 3. Temperatures optimized for these primers are shown in Table 5.

   After optimizing the primers and PCR conditions for amplification and sequencing, all 15 control samples were run for gradient PCR and their gel bands pictures were documented.

Table 5: Primers and PCR conditions for amplification and sequencing of exons 10, 11, 13, 14, 15 and 16.

Exons	PCR amplification	Annealing temperature standardized in the lab
10	GGGCCTATGCTTGCGACACCA	64.4^0C
	CCAGAGGGAGGGAGGGAAGTTT	
11	GGTCTAGGAGGGGGCAGTAAATGG	65.5^0C
	CAGCGTTGGCAGCCCCTCACAG	
13	AGAAGCCTCAAGCAGCATCGTC	63.3^0C
	AGGAGCAGTAGGGAAAGGGAGAAA	
14	ACGAGCAGCAGGAGGCAGAGA	67.4^0C
	GAGTGTGGCATGGTGGGGGAGTGG	
15	CCCCCGGCCCAGGTCTC	63.3^0C
	GCTCCACTAATCTTCGGTATCTTT	
16	GGCCTTCTCCTTTACCCCTCCTT	61.3^0C
	CAGCCATTTGCCTCACGAACCC	

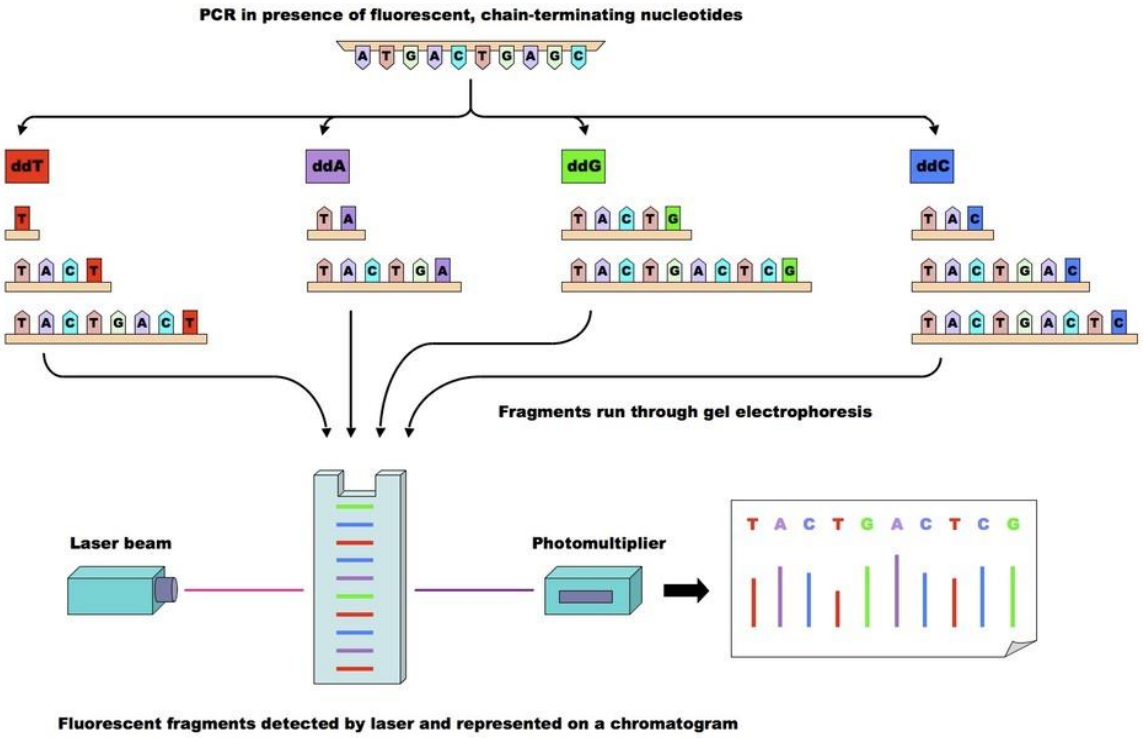


Fig 1: Diagrammatic representation of DNA sequencing by the Sanger method

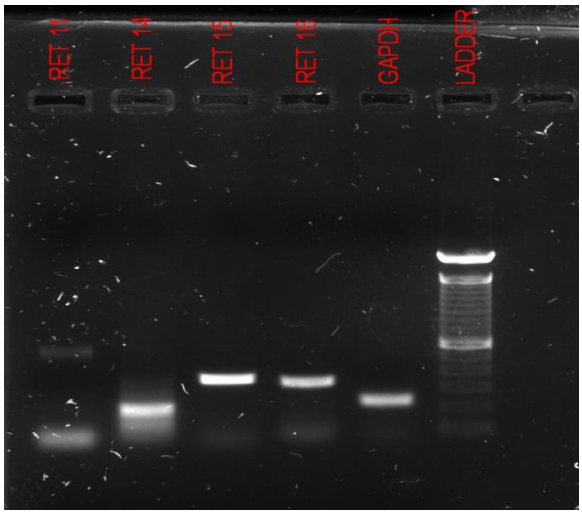


Fig 2: Bands of RET exons showing nonspecific bands at 55^0C

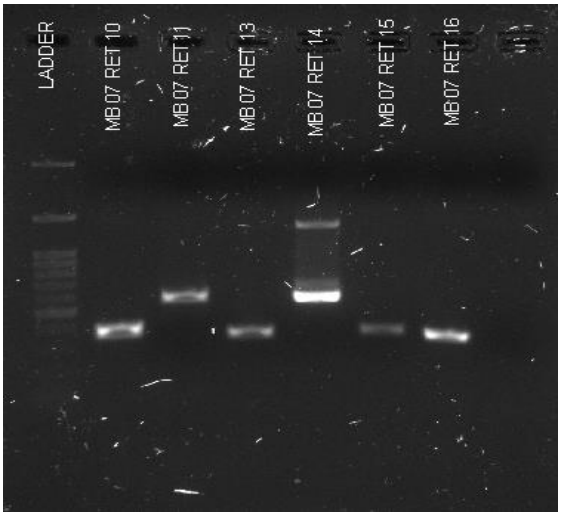


Fig 3: Bands of entire six exons RET gene is seen at Gradient temp. range of 60- 67^0C

2. Analysis of RET variants:

PCR amplification and sequencing for the exons 10 to 16 was performed to identify the mutations in the control and HD possible samples. The forward and reverse sequence was compared against the reference gene to find sequence changes. There were several nucleotide changes observed in both control and patient sample DNA. We identified the presence of D624N, G691S, L679L and S904S mutations in the sample analyzed (Table 6 and 7).

Table 6: List of variations in exon 10-16 seen in control group

Case No.	Position and amino acid change	Case No.	Position and amino acid change
C1	Exon 11 (G691S)	C9	Exon 15 (S904S)
C2	Exon 13 (L769L)	C10	No variation seen
C3	Exon 13 (L769L)	C11	Exon 13 (L769L)
C4	Exon 13 (L769L)	C12	No variation seen
C5	Exon 13 (L769L)	C13	No variation seen
C6	No variation seen	C14	Exon 13 (L769L)
C7	No variation seen	C15	Exon 13 (L769L)
C8	Exon 13 (L769L)		

Table 7: List of variations seen in 15 patients with Hirschsprung disease

Case. No	Range of aganglionic segment	Position and amino acid change	Case. No.	Range of aganglionic segment	Position and amino acid change
T1	RSHD	Exon 13 (L769L)	T9	LSHD	Exon 10 (D624N)
T2	RSHD	No variation seen	T10	LSHD	No variation seen
T3	RSHD	No variation seen	T11	TCA	Exon 13 (L769L)
T4	RSHD	Exon 13 (L769L)	T12	TCA	Exon 13 (L769L)
T5	RSHD	No variation seen	T13	TCA	Exon 13 (L769L)
T6	LSHD	No variation seen	T14	TCA	No variation seen
T7	LSHD	No variation seen	T15	TCA	No variation seen
T8	LSHD	No variation seen			

DNA Sequence Analysis:

Control Samples:

PCR amplification of exons 10 to 16 of RET gene from control group and sequencing of the PCR product suggest changes in some of the DNA samples. Analysis of electropherograms using Finch TV and Clustal W2 revealed that eight out of 15 control sample (C2, C3, C4, C5, C8, C11, C14, and C15), showed nucleotide alteration of c2307T>G in exon 13 (Fig 4 and 5). In addition, one of the control sample (C9) also showed nucleotide alteration of c2712C>G in exon 15 (Fig 6 and 7).

An additional alteration of nucleotide G>A was observed in exon 11 of RET gene (Fig 8 and 9) in C3. This alteration was seen at c2071 position.

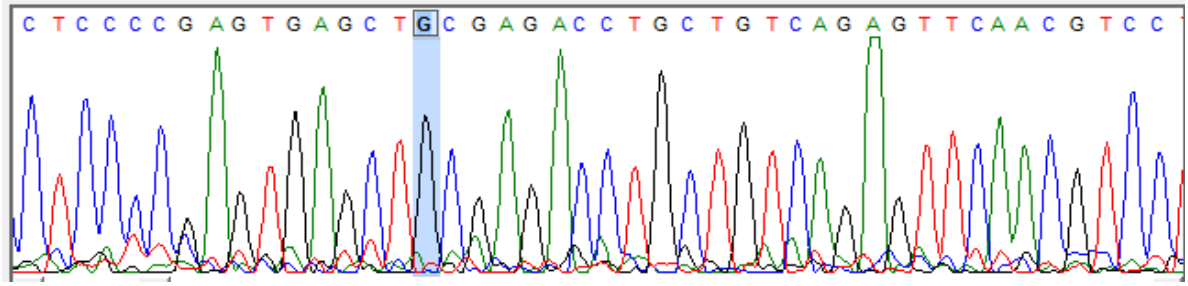
Test Samples:

Out of 15 confirmed HD cases, one case from long segment HD (T9) revealed alteration of G>A nucleotide in exon 10 of RET gene (Fig 10 and 11).

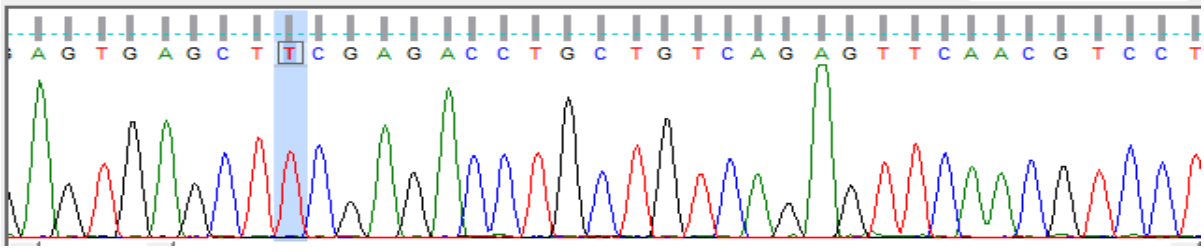
In addition, two cases from RSHD (T1 and T4) and three from TCA (T11, T12 and T13) also showed alteration of c2307T>G in exon 13. However, these variations were also seen in control samples.

```
CLUSTAL O (1.2.1) multiple sequence alignment


Subject RET 13      CATGGAAGGGGCTTCCAGGAGCGATCGTTTGCAACCTGCTCTGTGCTGCATTTCAGAGAA
Native RET 13       ------------------------------------------------------------AGAA
                                                                               ****

Subject RET 13      CGCCTCCCCGAGTGAGCTGCGAGACCTGCTGTCAGAGTTCAACGTCCTGAAGCAGGTCAA
Native RET 13       CGCCTCCCCGAGTGAGCTTCGAGACCTGCTGTCAGAGTTCAACGTCCTGAAGCAGGTCAA
                    ****************** ****************************************

Subject RET 13      CCACCCACATGTCATCAAATTGTATGGGGCCTGCAGCCAGGATGGTAAGGCCAGCTGCAG
Native RET 13       CCACCCACATGTCATCAAATTGTATGGGGCCTGCAGCCAGGATG---------------
                    *******************************************
```

Fig 4: Segment of the sequence of exon 13 of the *RET* gene (obtained from this study; subject) compared with the standard sequence of the exon 13 (Native). The highlighted change indicates the change at position 2307.



A. Electropherogram of exon 13 from control sample: c2307T>G alteration; homozygous variant.

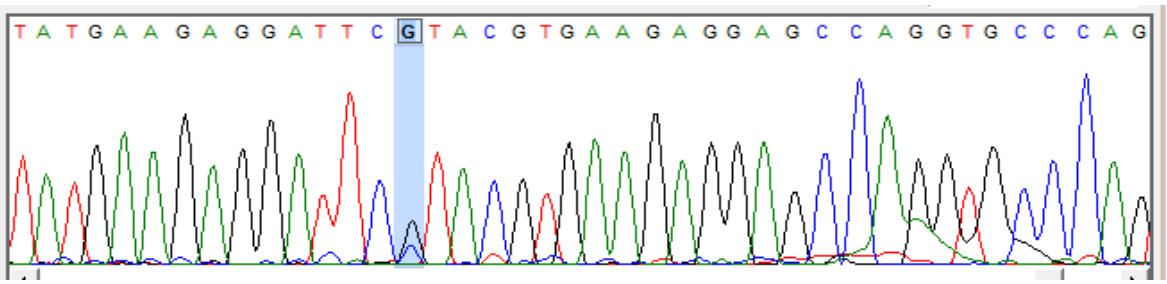


B. Electropherogram of exon 13 from control sample: no nucleotide alteration.

Fig 5: Control sample showing c2307T>G alteration at L679L in exon 13 of RET gene.

```
CLUSTAL O (1.2.1) multiple sequence alignment


Subject RET 15    CAGTGACCGCTGCTGCCTGGCCATGGCCTGACGACTCGTGCTATTTTTCCTCACAGCTCG
Native RET 15     ------------------------------------------------------------CTCG
                                                                               ****

Subject RET 15     TTCATCGGGACTTGGCAGCCAGAAACATCCTGGTAGCTGAGGGGCGGAAGATGAAGATTT
Native RET 15      TTCATCGGGACTTGGCAGCCAGAAACATCCTGGTAGCTGAGGGGCGGAAGATGAAGATTT
                   ************************************************************

Subject RET 15    CGGATTTCGGCTTGTCCCGAGATGTTTATGAAGAGGATTCGTACGTGAAGAGGAGCCAGG
Native RET 15     CGGATTTCGGCTTGTCCCGAGATGTTTATGAAGAGGATTCCTACGTGAAGAGGAGCCAG-
                  ************************************* ******************

Subject RET 15     TGCCCAGTCCCGGGGATGAGGCGGGGCTCCCAGGGATCCCAGGTGCACCATGCCGCAGGC
Native RET 15      -------------------------------------------------------------
```

Fig 6: Segment of the sequence of exon 15 of the *RET* gene (obtained from this study; subject) compared with the standard sequence of the exon 15 (Native). The highlighted change indicates the change at position 2712



A. Electropherogram of exon 15 from control sample: c2712C>G alteration; heterozygous variant.

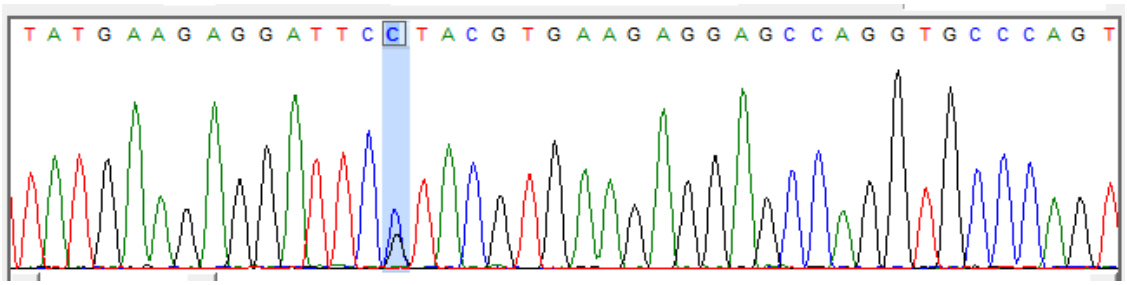
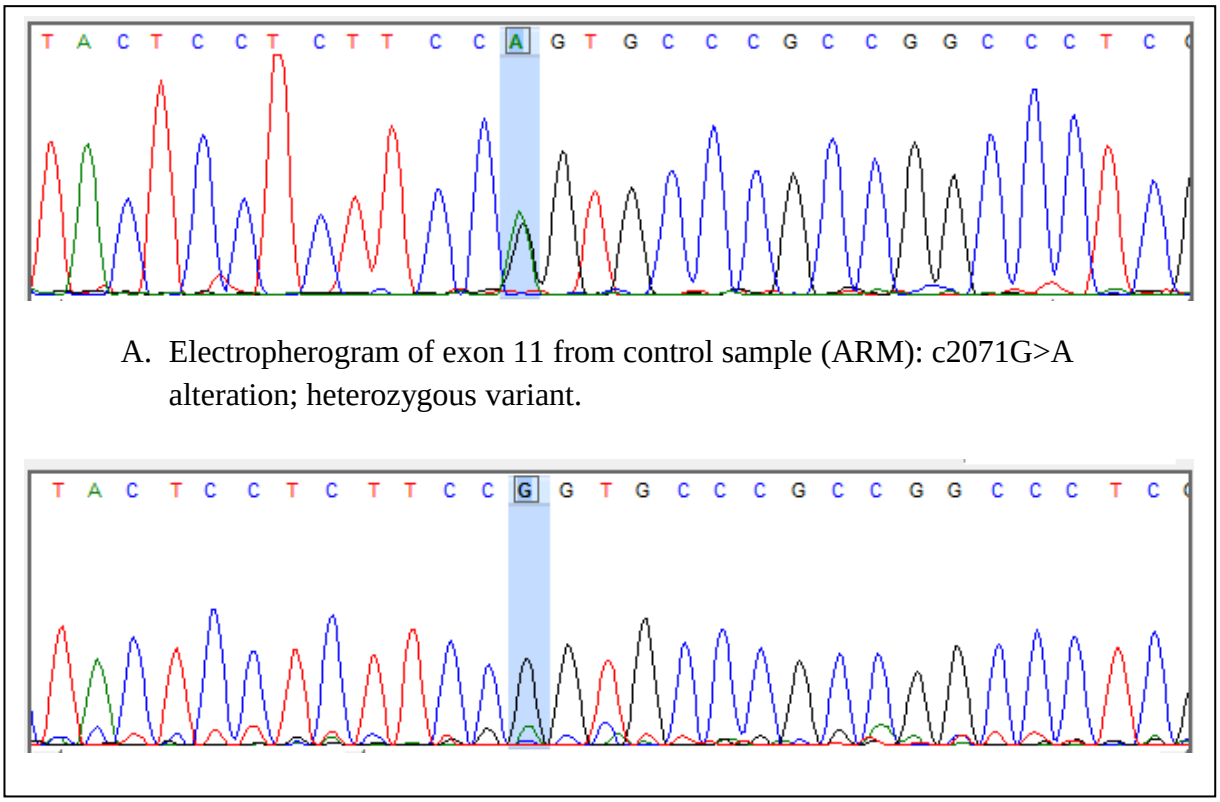


Fig 7: Control sample showing c2712C>G alteration at S904S in exon 15 of RET gene.

```
CLUSTAL O (1.2.1) multiple sequence alignment


Subject RET 11    ACCACCCCCACCCACAGATCCACTGTGCGACGAGCTGTGCCGCACGGTGATCGCAGCCGC
Native RET 11     -----------------ATCCACTGTGCGACGAGCTGTGCCGCACGGTGATCGCAGCCGC
                                    ****************************************

Subject RET 11    TGTCCTCTTCTCCTTCATCGTCTCGGTGCTGCTGTCTGCCTTCTGCATCCACTGCTACCA
Native RET 11     TGTCCTCTTCTCCTTCATCGTCTCGGTGCTGCTGTCTGCCTTCTGCATCCACTGCTACCA
                  ************************************************************

Subject RET 11    CAAGTTTGCCCACAAGCCACCCATCTCCTCAGCTGAGATGACCTTCCGGAGGCCCGCCCA
Native_RET11      CAAGTTTGCCCACAAGCCACCCATCTCCTCAGCTGAGATGACCTTCCGGAGGCCCGCCCA
                  ************************************************************

Subject RET 11    GGCCTTCCCGGTCAGCTACTCCTCTTCCAGTGCCCGCCGGCCCTCGCTGGACTCCATGGA
Native_RET11      GGCCTTCCCGGTCAGCTACTCCTCTTCCGGTGCCCGCCGGCCCTCGCTGGACTCCATGGA
                  *************************** ****************************

Subject RET 11    GAACCAGGTCTCCGTGGATGCCTTCAAGATCCTGGTGAGGGTCCCTGCGGGGCAGGGAAG
Native RET 11     GAACCAGGTCTCCGTGGATGCCTTCAAGATCCTG--------------------------
                  ********************************
```

Fig 8: Segment of the sequence of exon 11 of the *RET* gene (obtained from this study; subject) compared with the standard sequence of the exon 11 (Native). The highlighted change indicates the change at position 2071

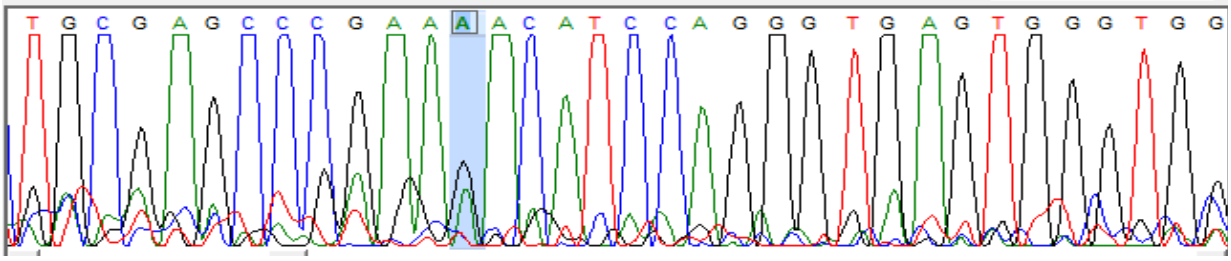


A. Electropherogram of exon 11 from control sample (ARM): c2071G>A alteration; heterozygous variant.

Fig 9: Control sample showing c2071G>A alteration at G691S in exon 11 of RET gene.

```
CLUSTAL O (1.2.1) multiple sequence alignment

Subject RET 10   GGGGGCAGGGTCTCGTGTGGACTGCCCTGGAATATGGGCGCCTGGGGTGGTCAGGCGCCC
Native RET  10   ------------------------------------------------------------

Subject RET 10   CAGGAGGCTGAGTGGGCTACGTCTGCCCTCAGGGGGCAGCATTGTTGGGGGACACGAGCC
Native RET  10   --------------------------------GGGGCAGCATTGTTGGGGGACACGAGCC
                                                 ****************************
Subject RET 10   TGGGGAGCCCCGGGGGATTAAAGCTGGCTATGGCACCTGCAACTGCTTCCCTGAGGAGGA
Native RET  10   TGGGGAGCCCCGGGGGATTAAAGCTGGCTATGGCACCTGCAACTGCTTCCCTGAGGAGGA
                 ************************************************************
Subject RET 10   AAAGTGCTTCTGCGAGCCCGAAAACATCCAGGGTGAGTGGGTGGCGGCCGGGACCACCAC
Native RET  10   GAAGTGCTTCTGCGAGCCCGAAGACATCCAGG----------------------------
                 ********************* *********
Subject RET 10   CACCTCCCAGCCCCATAGAGGTCTCAACAGAACATCTGAGGTCCCAACAAGGGAGGAGAT
Native RET  10   ------------------------------------------------------------
```

Fig 10: Segment of the sequence of exon 10 of the *RET* gene (obtained from this study; subject) compared with the standard sequence of the exon 10 (Native). The highlighted change indicates the change at position 1870



A. Electropherogram of exon 10 from LsHD): c1870G>A alteration; heterozygous variant.

B. Electropherogram of exon 10 from control sample: no nucleotide alteration.

Fig 11: LsHD showing c1870G>A alteration at D624N in exon 10 of RET gene.

A) Protein Structure Prediction using bioinformatics:

Conversion of DNA sequence to amino acid sequence:

The protein structure was analyzed to understand the effect of identified nucleotide alterations. The DNA sequences obtained from the electropherograms were converted into amino acid sequences using the EXPASY convert tool. The amino acid sequences were then compared with the standard amino acid sequences of the respective exons 10 to 16. The two amino acid sequences were then aligned to detect the changes.

There were no changes observed in the amino acid sequence of the patient sample. The Leucine at codon position 676 (Fig 12) and Serine at 904 (Fig 13) remained unchanged as compared to the reference amino acid sequence. Eight of the controls samples, 2 RSHD and 3 TCA cases have shown the nucleotide alteration of c2307T>G at codon 676 position in exon 13. One control sample also showed nucleotide alteration of c2712C>G at codon 904 position in exon 15, however this variation was not seen in any of the HD patients. Hence, these two variations may not be significant to the cohort.

Interestingly one of the control cases (C3) which had shown alteration of c2071G>A in exon 11 had led to the transition of Glycine to Serine at 691 codon position (Fig 14). Since this variation (G691S at c2071G>A) is not a silent mutation, still, its presence in the C3 was intriguing. It thus triggered us to reanalyze clinical and histopathological reports. And we found that C3 was a case of anorectal malformation (ARM).

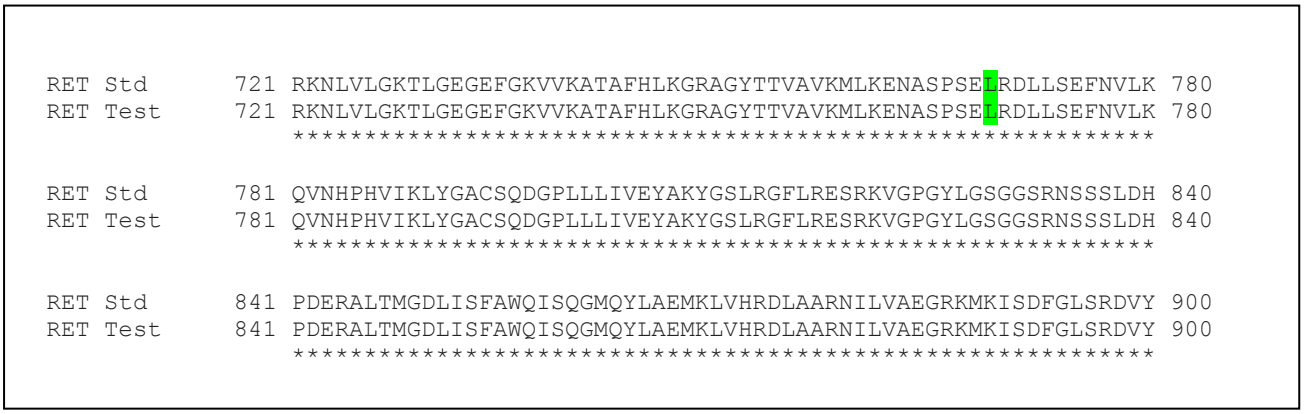


Fig 12: Amino acid sequence of exon 13 (obtained from this study; test) compared with the standard sequence of the exon 13 (Native) showing no change in Leucin at codon position 767.

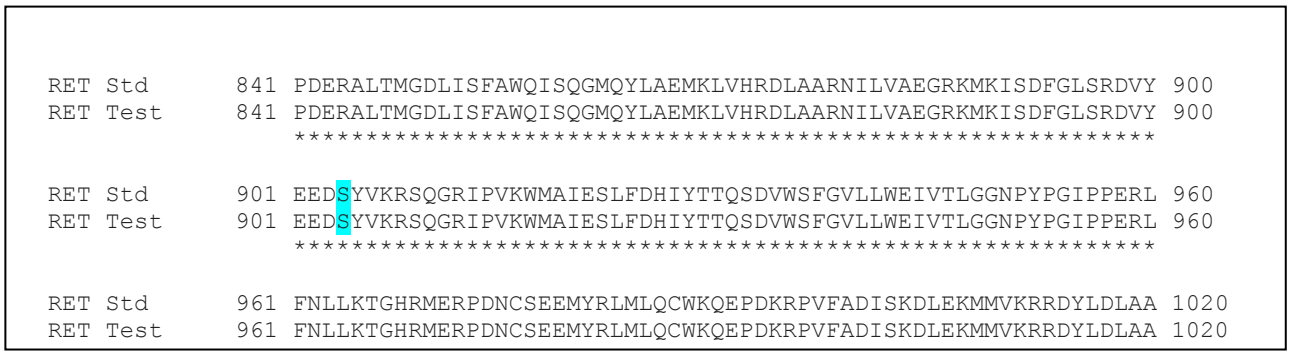


Fig 13: Amino acid sequence of exon 15 (obtained from this study; test) compared with the standard sequence of the exon 13 (Native) showing no change in Serine at codon position 904.

Fig 14: Amino acid sequence of exon 11 (obtained from this study; test) compared with the standard sequence of the exon 11 (Native) showing transition of Glycine to Serine at codon position 691.

A similar analysis of the mutation observed in the T9, LSHD patient showed a transition from Aspartic acid to Asparagine at 624 codon position (as shown in figure 15). This mutation was exclusively present in the LSHD and was not observed in the control sequence. Interestingly there is no report implicating or suggesting the existence of this mutation in other cohorts. Also, there is no evidence of the existence of this mutation in the Human Gene Mutation Database. This observation indicates the probable existence of a novel mutation in the present cohort.

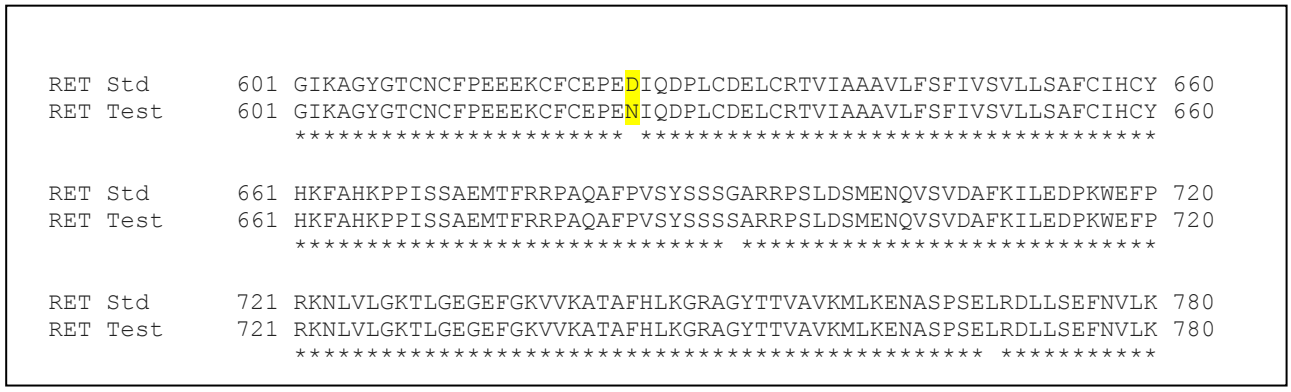


Fig 15: Amino acid sequence of exon 11 (obtained from this study; test) compared with the standard sequence of the exon 10 (Native) showing Aspartic acid to Asparagine at 624 codon position.

Glycine has a side chain of non-polar aliphatic residues whereas serine has a polar and non-charged side chain. Similarly, Aspartic acid has a negatively charged side chain and Asparagine has a polar, non-charged side chain. Alterations in these amino acids of different group may alter the protein structure of RET gene. Hence phyre 2 software was used to model the altered protein structure.

B) Protein Modelling

The amino acid sequences were analysed for the secondary and tertiary structural forms of the protein using Phyre 2 software.

Due to alteration in amino acids G691S [in one of the control sample] and D624N [in LSHD], the possible secondary and tertiary structural changes of the **RET** protein is represented in the form of alpha helixes and beta sheets. (Fig 16 - 21). There were no significant changes observed in the secondary structure because of the alteration in amino acids D624N in exon 10 of RET gene from LSHD (Fig 16 and 17). As shown in figure 17, we found no alteration in alpha helix and beta strands. Analysis of the tertiary structure also indicated that there was no significant change in the structure of the protein due to the change in the amino acid at the positions 624 (Fig. 18).

Similarly, we also found, the alteration in amino acid G691S in exon 11 from a control group (a case of ARM) has no major effect in the secondary structure of RET protein (Fig. 19 and Fig. 20). As shown in fig. 20, we observed a minor shift in alpha helix from 26% to 25% and beta strands from 17% to 19%, however, these changes are not significant. Tertiary structural analysis of the RET gene also indicated that there was no significant change in the structure of the protein due to the change in the amino acid at the positions 691 (Fig. 21).

Fig 16: Secondary structure of the protein of a portion of the exon 10 of the *RET* gene. **Red** arrows indicate the change from Aspartic acid (1) to Asparagine (2).

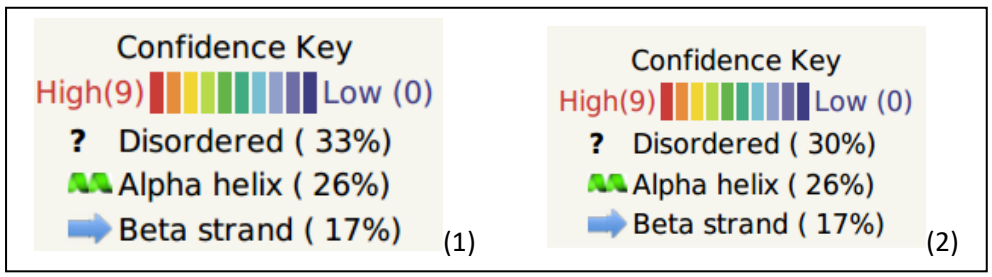


FIG 17: Confidence Keys to secondary structure of the protein (exon 10 of RET gene) of the (1) normal/ standard sequence and of (2) patients with homozygous variant status obtained from Phyre-2

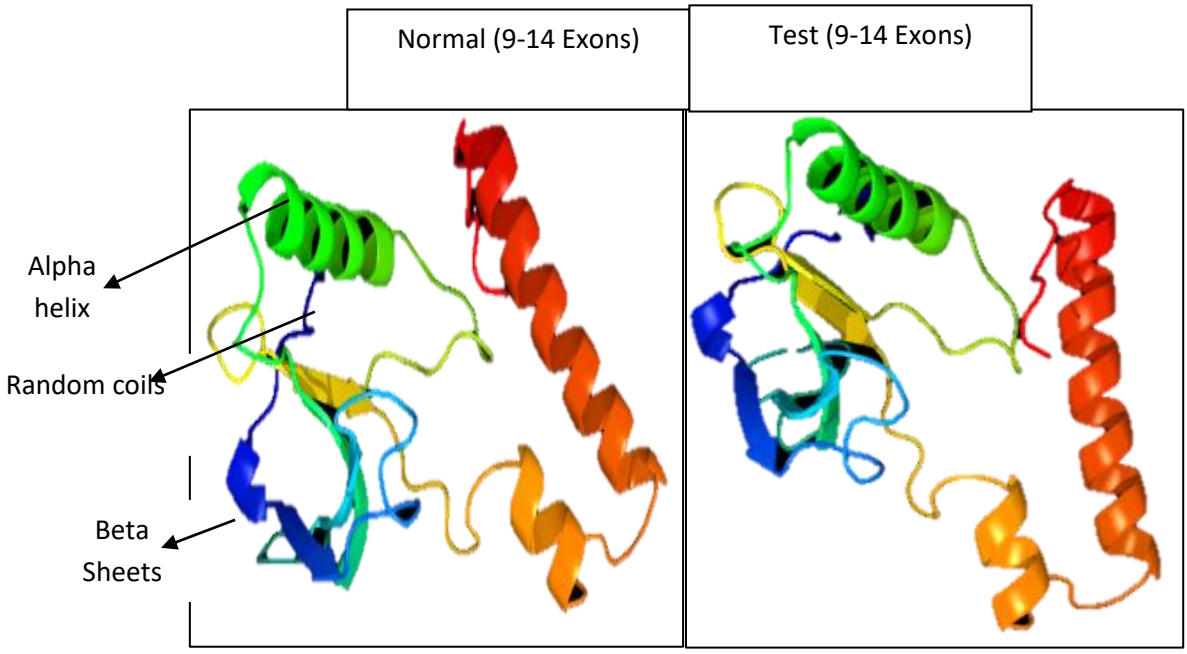


Fig 18: Parts of the secondary structure of a protein (exon 9-14 of RET gene shown here, generated using the sequence obtained from this study) for D624N mutations in exon 10.

Fig 19: Secondary structure of the protein of a portion of the exon 11 of the *RET* gene. **Red** arrows indicate the change from Glycine (1) to Serine (2).

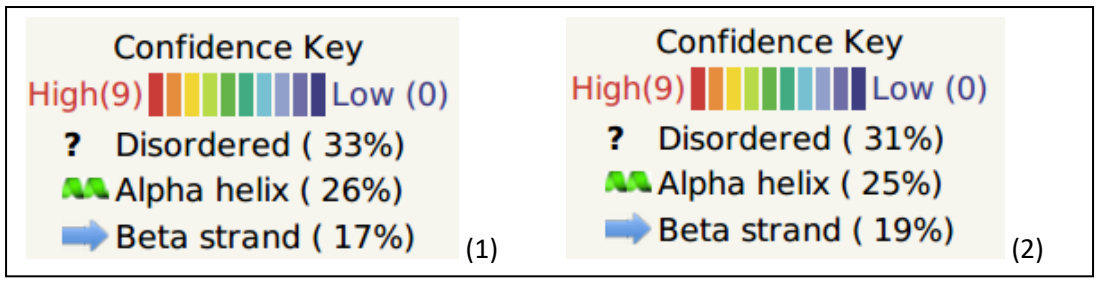


FIG 20: Confidence Keys to secondary structure of the protein (exon 11 of RET gene) of the (1) normal/ standard sequence and of (2) patients with homozygous variant status obtained from Phyre-2.

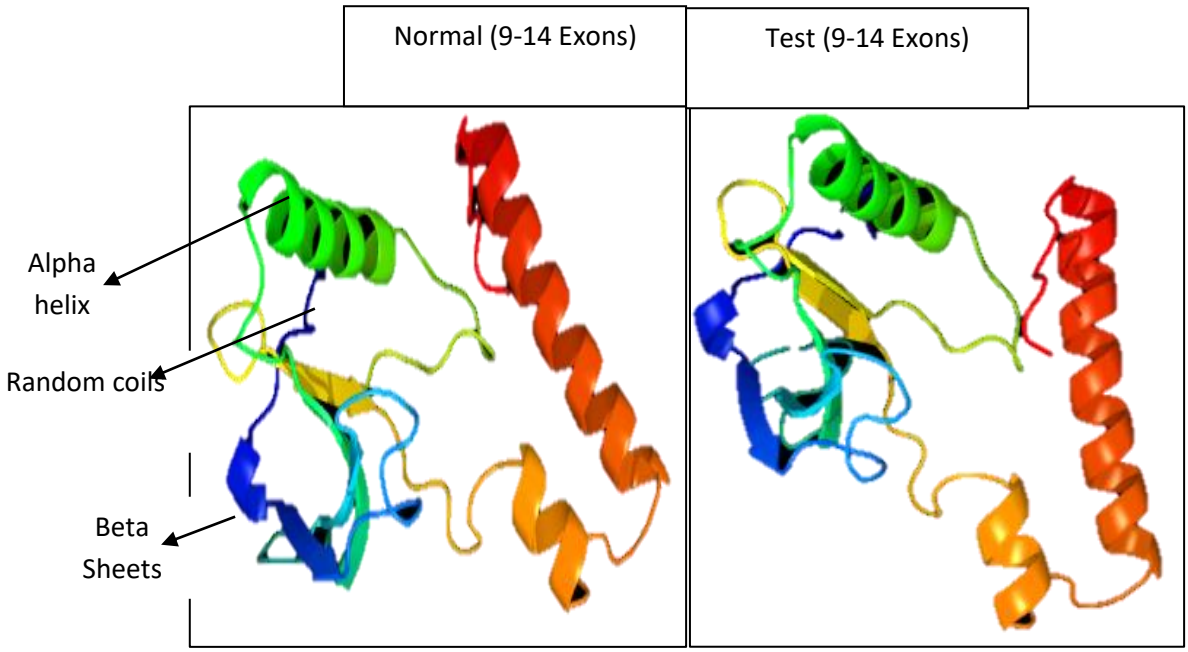


Fig 21: Parts of the secondary structure of a protein (exon 9-14 of RET gene shown here, generated using the sequence obtained from this study) for G691S mutations in exon 11.

DISCUSSION:

One case of long segment HD revealed alteration of G>A nucleotide in exon 10 of RET gene. This alteration was seen at c1870. It also caused transition of Aspartic acid to Asparagine at 624 codon position and hence it is not a silent mutation. This mutation (D624N) at c1870G>A) was not observed in our control samples nor did we find any evidence in The Human Gene Mutation Database. Hence, it suggests that this might be a novel mutation in our cohort.

In contrast, Angrist et al. (22) have reported Cys 609 Tyr variation in familial and Cys 620 Arg variation in sporadic cases of HD in exon 10 of RET gene. Decker et al. (24) have also reported these two mutations in RSHD along with Cys 618 Ser in RSHD and LSHD, Cys 620 Trp and Cys 609 Tyr in RSHD.

An additional alteration of nucleotides G>A was observed in exon 11 of RET gene in one of our control sample. This alteration was seen at c2071 position leading to transition of Glycine to Serine at 691 codon position. Since, this variation (G691S at c2071G>A) is not a silent mutation and seen in control sample, it triggered us to reanalyze clinical and histopathological reports. And we found that this control sample was a case of anorectal malformation (ARM).

This mutation was unexpected and hence, we also looked for the novelty of this mutation in ARM using **The Human Gene Mutation Database** and various literature for ARM. We found, it has been reported as SNP by Wu et al (27) and Garcia-Barceló et al., (25) in both control and HD patients. Wu et al., have also found no significant differences in its allele distribution between HD, ARM and in normal control. Knowles et al., (28) have also reported this variation in HD. Along with G691S variation, Garcia-Barceló et al., have also reported P679P; c2307T>G in exon 11 of HD patients in Chinese population. Sakai et al., (26) have

found T654A; c1906G>A in exon 11 of HD patients in Japanese population. Attie et al., (23) have shown S690P variation in French HD patients. These variations were not reported in our study.

Eight of our control samples showed L679L variation with nucleotide alteration of c2307T>G in in exon 13. This variation was also seen in two cases of RSHD and in three cases of TCA. Knowles et al. (28) have also found this variation with HD in English population. Sakai et al. (26) have conducted mutation analysis in sporadic HD patients in Japanese population and found L679L variation exon 13. Similarly, Garcia-Barceló et al. (25) in Chinese patients with sporadic HD, Wu et al. (27) in Taiwan population and Ishii et al. (4) in Japanese population have also found L679L variation with nucleotide alteration of c2307T>G in in exon 13 of RET gene. Ishii et al. (4) have found this variation in 11.1% of HD patients including RSHD, LSHD and in TCA and in 60% of control population whereas this study report the presence of this mutation in 55.34% of control and in 33.34% in HD patients including RSHD and TCA. Along with this variation Ishii et al., (4) have also found V778D variation with nucleotide alteration of c2333T>A in exon 13 of RET gene in 90% of control and in 66.7% of RSHD. Attie et al., (23) have reported S767R variation in exon 13 of sporadic case of LSHD but none of these two mutations, L679L and V778D, were seen in this study.

One of the control samples also showed S904S variation with nucleotide alteration of c2712C>G in exon 15. However, this variation was not seen in any of the 15 HD patients. Garcia-Barceló et al. (25) have reported this variation in 4.5% HD patients and in 10.5% control group in Chinese population whereas Wu et al. (27) have found no significant differences in its allele distribution between HD, ARM and in normal control in Taiwan.

Several other mutations have been reported such as Serine 899 variation in exon 15, R873Q, F893L and K907E. Knowles et al. (28) have reported the existence of Serine 899 variation in

exon 15 in the English population. Attie et al. (23) have shown R873Q, F893L and K907E variations in exon 15 in French population of LSHD. However, we have not seen any of these variations.

CONCLUSION:

Sequencing of six exons namely, 10, 11, 13, 14, 15 and 16 of RET gene in 30 samples comprising of tests (Hirschsprung disease) and control (normal) of Indian subpopulation in this pilot study reports the occurrence of a novel mutation, D624N in exon 10 of a single patient with LSHD. However, majority of reported mutations in other population were not seen in our population. This could be because of a small sample size and limited number of exons scanned. Hence, this calls for complete RET gene sequencing in large number of sample to identify specific mutations in other exons/introns.

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SUMMARY

The present study titled ***" Biology of Hirschsprung Disease: Pathomorphological, Histochemical, Immunohistochemical and Genetic (RET Gene) Study of the Enteric Nervous System"*** is a prospective cross-sectional study on patients from the Indian subpopulation with Hirschsprung disease over three and a half years (Nov 2011 to June 2015) at the Department of Pathology, St. John's Medical College, Bangalore - a national referral centre for diagnosis of Hirschsprung disease in India.

The study with five chapters has explored pathobiology of Hirschsprung disease (HD) in the Indian context with emphasis on rapid reliable user friendly diagnostic modalities and also made an attempt at investigating the molecular basis of the HD to gain insight into its possible pathogenic mechanisms. Their summary is as follows:

1. The rapid modified agar-paraffin block technique designed in the first study titled *"Improvised double-embedding technique of minute biopsies: A mega boon to histopathology laboratory"* has revolutionized the processing of multiple minute mucosal and seromuscular biopsies which mandate proper orientation to visualize neuronal plexuses, especially when sampled from neonates. The simple reliable user friendly method has improved the quality of diagnostic information by optimal orientation, better quality of sections, faster turnaround time, cost-effectiveness by economizing on the number of paraffin blocks, manpower, chemical reagents and laboratory infrastructure. The modified tissue blocks are also best suited for enzyme and immunohistochemistry in addition to routine histochemistry.
2. The second study titled *"Improvised rapid Acetylcholinesterase histochemistry versus calretinin immunohistochemistry in the evaluation of colorectal biopsies for*

Hirschsprung disease" evaluated calretinin, a Vitamin D dependent calcium binding protein expressed in central and peripheral neural system, on formalin fixed biopsies and compared the results with the improvised modified rapid AChE histochemistry (designed in the Department) on their corresponding fresh rectal biopsies taken for the primary diagnosis of HD. Calretinin proved as a reliable immune marker in ruling out the diagnosis of HD on formalin fixed rectal mucosal biopsy by highlighting granular staining of intrinsic fibres in the mucosa and submucosa in suspect cases of HD. The study also proved that the accuracy of diagnosis in Hirschsprung disease could be improved by employing both AChE and Calretinin stains.

3. The detailed evaluation of Synaptophysin immunohistochemistry as a labelling immunohistochemical method in the third study titled *"Role of Synaptophysin in the Intra-Operative Assessment of Quadrantic Innervation of the Proximal Doughnut in Hirschsprung Disease"* assessed proximal doughnut for innervation abnormalities intraoperatively to find its suitability for anastomosis for pull through surgeries. The marker specific for the synaptic vesicles in the central and peripheral nervous system and the main constituent of AChE storage compartments, and an important neuromuscular junction marker, highlighted the morphology of ganglion cells, indirectly reflected their functional status by demonstrating synapses at the level of muscle fibers on frozen sections and mapped the ganglionic –aganglionic interface with the pattern and intensity of the SY-positive fibre distribution in the muscularis propria.
4. The forth study *"The quest for a positive diagnostic marker for Hirschsprung Disease in formalin fixed rectal biopsies: A detailed seven marker IHC study"* describes in detail the hunt for a positive diagnostic marker on formalin fixed rectal

biopsy in the diagnosis for HD from among the panel of seven neural markers namely Calretinin, GFAP, Synaptophysin, PGP 9.5, CD 56, NF and S100. None of these markers specifically stained and differentiated the hyperplastic-hypertrophic nerve bundles of Hirschsprung disease from the normal nerve bundles and extrinsic serosal nerves. Though CD 56 and S-100 failed to stain ganglion cells, they were not specific for hypertrophic nerve bundles and hence, these markers could not be considered as markers for HD. Thus, the quest of a novel marker for abnormal enteric nervous system continues with the proposal for the next panel of markers which may attempt to highlight pathology in perineurium.

5. The fifth study *"Diversity of RET Proto-oncogene Mutation in an Indian sub population of Hirschsprung disease: A Pilot Study "* highlights the association of RET gene in Hirschsprung disease. Sequencing of six exons namely, 10, 11, 13, 14, 15 and 16 of RET gene in 30 samples comprising of tests and control of Indian subpopulation in this pilot study reports the occurrence of a novel mutation, D624N in exon 10 of a single patient with LSHD. The variations seen in RSHD, and TCA were also seen in the control group. However, the reported mutations in other study population were not seen in the limited samples studied here and this could also because of limited number of exons scanned. Hence, this calls for complete RET gene sequencing of a large sample size to strengthen the data and to study its relevance.

CONCLUSION

This thesis titled ***"Biology of Hirschsprung Disease: Pathomorphological, Histochemical, Immunohistochemical and Genetic (RET Gene) Study of the Enteric Nervous System"***, a prospective cross-sectional study comprising of systematic morphological, enzyme histochemical and immunohistochemical approach using improvised modified rapid acetylcholinesterase (AChE) histochemistry and appropriate panel of neural markers on rectal biopsies carried out on Indian population has drastically improvised diagnosis of Hirschsprung disease and has guided surgeons for better patient/surgical management. This maiden study which included various rapid tissue diagnostic modalities has outcome relevant to histopathology centres of developing countries like India.

The molecular pilot study done on Hirschsprung disease patients done for the first time in India has helped to get preliminary data on a limited sample size. The results are not statistically significant owing to small number of samples and hence, mandates a whole RET gene sequencing of a sample size required to strengthen the data obtained and study its relevance.

PUBLICATIONS

1. **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: 369-75.
2. **Yadav L**, Thomas S, Kini U. Improvised double- embedding technique of minute biopsies: A mega boon to histopathology laboratory. Indian J Pathol Microbiol 2015;58:12-6.
3. **Yadav L**, Babu M. K, Das K, M S, Puttegowda D, Gowri S. Role of synaptophysin in the Intra-Operative Assessment of Quadrantic Innervation of the Proximal Doughnut in Hirschsprung Disease. Natl. Med. J. India.2016 (accepted for publication).
4. Mohanty S, Kini U, Das K, Puttegowda D, **Yadav L**, Babu MK, Mahadevappa K, Kumar P, Mahadevaiah SA, Deb M. Appendicular Biopsy in Total Colonic Aganglionosis: A Histologically Challenging and Inadvisable Practice. Pediatr Dev Pathol. 2016 Sep 9. [Ahead of Print]

APPENDICES

I. Institutional Ethical Clearance Certificates [A-C]

II. Patient information sheet.

III. Consent form

IV. Standardized operating procedure for rapid H & E staining technique.

V. Standardized operating procedure for rapid acetylcholinestrase (AChE) staining technique

VI. Staining protocol of immunohistochemistry (IHC).

VII. Standard Operating Procedure for DNA Extraction from EDTA Blood Sample using QIAMP DNA Mini Kit.

VIII. Abstract of "**Improvised double-embedding technique of minute biopsies: A mega boon to histopathology laboratory**" for oral presentation at the 60th Annual PPS Meeting (Jointly with SPP), 4th – 6th September 2014 at International Conference Center, Birmingham, UK.

IX. Abstract of **"Calretinin immunohistochemistry versus modified rapid Acetylcholinesterase histochemistry in the evaluation of colorectal biopsies for Hirschsprung disease"** for oral presentation at the 60th Annual PPS Meeting (Jointly with SPP), 4th – 6th September 2014 at International Conference Center, Birmingham, UK.

X. Abstract on **"Role of Synaptophysin in leveling circumferential full thickness (Doughnut) bowel biopsy in Hirschsprung disease"** for oral presentation at the 61 Annual Conference of Indian Association of Pathologists and Microbiologists, 2012 held at Jamnagar, 14- 16th Dec. 2012.

XI. Acknowledgement of paper titled **"Role of Synaptophysin in leveling circumferential full thickness (Doughnut) bowel biopsy in Hirschsprung disease"** accepted for publication in The National Medical Journal of India.

APPENDIX- I A

ST. JOHN'S MEDICAL COLLEGE & HOSPITAL
INSTITUTIONAL ETHICAL REVIEW BOARD

No : IERB/1/876/2011

20th December 2011

Dr. Usha Kini
Professor
Dept. of Pathology
St. John's Medical College
Bangalore – 560 034.

IERB Study Ref No. 201 / 2011

Dear Doctor,

Sub : Approval of Research proposal by the I.E.R.B.

I wish to inform you that your Research Project entitled, **"Biology of Hirschsprung disease : pathomorphological cytochemical, immunohistochemical and genetic (RET GENE) study of the enteric nervous system** has been approved by the Institutional Ethical Review Board, SJMC & H, on **7th December 2011.** The approval of I.E.R.B. is valid for a period of 2 years from the date of approval given.

You must inform the IERB of the following:

1. The Occurrence of Serious Adverse Events/Drug Reactions and/or Death, while conducting this Trial in the specified format.
2. Protocol amendment in the specified format
3. a) Discontinuation (b) Abandonment (c) Completion of this Trial, stating the reasons, if the situation of 2(a) or 2(b) is encountered.
4. (a) It is mandatory that a 6 monthly Interim Review Report on the status of the project be submitted to the Convenor in the specified format.
   (b) On completion of the above Research Project – the Principal Investigator is responsible for submitting a brief summary of the results obtained, to the Convenor of the Institutional Ethical Review Board. (I.E.R.B.) through the Chairperson / Dean.

With best wishes,

Rev. Fr. Shaji Kochuthara, Ph.D.,
Chairperson

CHAIRPERSON
Institutional Ethical Review Board
St. John's Medical College & Hospital
Sarjapur Road
Bangalore - 560 034, India.

CC: The Associate Director, SJMC
The Medical superintendent, SJMCH
The HOD for file

INSTITUTIONAL ETHICAL REVIEW BOARD ★ ST. JOHN'S MEDICAL COLLEGE & HOSPITAL ★ BANGALORE-560 034. INDIA.

Institutional Ethical Review Board
Ground Floor, St. John's Medical College, Sarjapur Road, Bangalore - 560 034, India
Telephone : 22065780 Fax : 25634123 E-mail: sjmcierb@gmail.com

APPENDIX- I B

ST. JOHN'S NATIONAL ACADEMY OF HEALTH SCIENCES
INSTITUTIONAL ETHICS COMMITTEE

No : IEC/1/359/2014

26th May 2014

Dr. Usha Kini
Professor
Dept. of Pathology
St. John's Medical College
Bangalore – 34.

IERB Study Ref.No.201 / 2011

Dear Doctor,

Ref : Study titled "**Biology of Hirschsprung Disease : pathomorphological, cytochemical, immunohistochemical and genetic (RET GENE) study of the Enteric Nervous System**"

Sub : Extension of Approval vide letter dated 28th April 2014

Following the Institutional Ethics Committee (IEC) meeting held on 8th May 2014, your request for extension of approval for your study entitled "**Biology of Hirschsprung Disease : pathomorphological, cytochemical, immunohistochemical and genetic (RET GENE) study of the Enteric Nervous System**" has been favourably considered and the approval has been extended further for a period of **ONE YEAR from 8th May 2014.**

In case the study needs renewal of approval, please **apply for a renewal by March 2015.**

You are requested to submit the interim reports periodically and study related documents to the IEC.

With Best Wishes!

Dr. Rema Devi, MS, DNB.,
Member Secretary
Institutional Ethics Committee

MEMBER SECRETARY
Institutional Ethics Committee
St. John's National Academy of Health Sciences
Sarjapura Road,
Bangalore-560 034, India.

Institutional Ethics Committee
1st Floor, Zablocki Learning Center (St. John's Library), St. John's Medical College, Sarjapur Road
Bangalore - 560 034, India. Telephone : (080) 25634123 / 49466346 E-mail: sjmcierb@gmail.com

APPENDIX- I C

ST. JOHN'S NATIONAL ACADEMY OF HEALTH SCIENCES
INSTITUTIONAL ETHICS COMMITTEE

No : IEC/1/419/2015

20th April 2015

Dr. Usha Kini
Professor
Dept. of Pathology
St. John's Medical College
Bangalore – 34.

IEC Study Ref.No.201 / 2011

Dear Doctor,

Ref : Study titled "**Biology of Hirschsprung Disease : pathomorphological, cytochemical, immunohistochemical and genetic (RET GENE) study of the Enteric Nervous System**"

Sub : Extension of Approval vide letter dated 12th March 2015

Following the Institutional Ethics Committee (IEC) meeting held on 9th April 2015, your request for extension of approval for your study entitled "**Biology of Hirschsprung Disease : pathomorphological, cytochemical, immunohistochemical and genetic (RET GENE) study of the Enteric Nervous System"** has been favourably considered and the approval has been extended further for a period of **TWO YEARS from 8th May 2015 to 7th May 2017.**

In case the study needs renewal of approval, please **apply for a renewal by March 2017.** You are requested to submit the interim reports periodically and study related documents to the IEC.

With Best Wishes!

Dr. Rema Devi, MS, DNB.,
Member Secretary
Institutional Ethics Committee

MEMBER SECRETARY
Institutional Ethics Committee
St. John's National Academy of Health Sciences
Sarjapura Road,
Bangalore-560 034, India.

Institutional Ethics Committee
1st Floor, Zablocki Learning Center (St. John's Library), St. John's Medical College, Sarjapur Road
Bangalore - 560 034, India. Telephone : (080) 25634123 / 49466346 E-mail: sjmcierb@gmail.com

APPENDIX- II

Patient Information Sheet:

What is Hirschsprung disease (HD)?

Hirschsprung disease (HD) is a disease of the large **intestine** that causes severe **constipation** or intestinal **obstruction**. Constipation means stool moves through the intestines slower than usual. Bowel movements occur less often than normal and stools are difficult to pass. Some children with HD can't pass stool at all, which can result in the complete blockage of the intestines, a condition called intestinal obstruction. People with HD are born with it and are usually diagnosed when they are infants. Less severe cases are sometimes diagnosed when a child is older. An HD diagnosis in an adult is rare.

How is HD diagnosed?

HD is diagnosed based on symptoms and test results.

A doctor will perform a physical exam and ask questions about your child's bowel movements. HD is much less likely if parents can identify a time when their child's bowel habits were normal.

If HD is suspected, the doctor will do one or more tests.

Biopsy

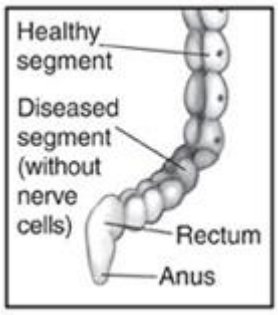
Biopsy is the most accurate test for HD. The doctor removes a tiny piece of the large intestine and looks at it with a microscope. If nerve cells are missing, HD is the problem.

How is HD treated?

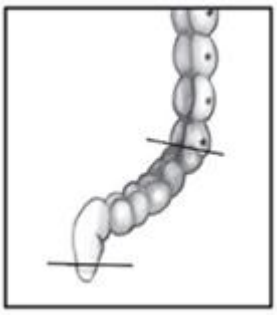
Pull-through Procedure

HD is treated with surgery called a pull-through procedure. A surgeon removes the segment of the large intestine lacking nerve cells and connects the healthy segment to the anus. The pull-through procedure is usually done soon after diagnosis.

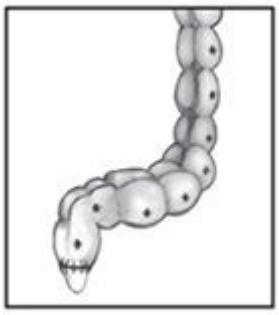
Pull-through Procedure



Before pull-through surgery: The diseased segment doesn't push stool.



Step 1: The diseased segment is removed.



Step 2: The healthy segment is attached to the remaining rectum.

Points to Remember

- Hirschsprung disease (HD) is a disease of the large intestine that causes severe constipation or intestinal obstruction. People with HD are born with it.
- The large intestine, which includes the colon and rectum, is the last part of the digestive tract.
- The cause of HD is unclear. HD is not caused by anything a mother did while pregnant.
- The main symptoms of HD are constipation or intestinal obstruction, usually appearing shortly after birth.
- Newborns with HD almost always fail to have their first bowel movement within 48 hours after birth.
- HD is diagnosed based on symptoms and test results.
- HD is treated with surgery called a pull-through procedure.
- A child who has been very sick from HD may need an ostomy to get better before the pull-through procedure.

- **What about when Hirschsprung disease occurs with other health problems?**

  Hirschsprung disease is the only health concern in the majority of people who have it. However, about 30% of people with Hirschsprung disease were born with additional health problems. If that is the case, it may be due to chance occurrence, or the person may have a "syndrome". A syndrome is a combination of birth defects and/or health problems that have the same underlying genetic cause. If a syndrome is suspected, the person with HSCR should be seen by a geneticist (a genetics doctor). The chance of recurrence in future children may be affected by whether or not a syndrome is present.

- **Is there genetic testing available for Hirschsprung disease?**

  Yes, but it is only available for one of the genes found to play a role in HD. This is the RET gene--the main gene implicated in Hirschsprung disease. It is estimated that a genetic change (mutation) will be found in the RET gene in about 15-35% of isolated cases (individuals with no family history), and about 50% of individuals with a family history of Hirschsprung disease. For long segment and total colonic Hirschsprung disease, a mutation will be found in about 70-80% of cases. In short segment disease, the likelihood of finding a mutation is generally lower, about 10-35%.

Getting Involved

If you or your family member has been diagnosed with Hirschsprung disease (HD), we would welcome your participation in our research study! We need participants with all segment lengths of Hirschsprung disease, with or without a family history of the disease, and with or without other health problems. Those who are diagnosed with the Hirschsprung disease and get enrolled in the study, the rectal or leveling biopsy received for the same will be further investigated for marker studies using

immunohistochemistry. About 3ml of peripheral blood will be collected for mutations with reference to RET gene and if associated with Downs', will be subjected for GTG banding technique, so as to associate the results with length of aganglionosis and response to surgical treatment.

For further details, one can contact Dr Usha Kini at 9448270384 or Dr Kanishka at 9844055045**. Or IERB Convenor at 080-49466346/25634123.**

APPENDIX- III

St. John's Medical College, Bangalore
Department of Pathology, Paediatric Surgery and Division of Cytogenetics-Dept. of Anatomy
Biology of Hirschsprung disease
CONSENT FORM

Name of the patient .....Hospital. / IP Nos.....

Surgery proposed: Rectal biopsy, colonic biopsy & colostomy/pull through / venepuncture for genetic studies

I hereby confirm that I have been informed in the language understood by me of the risks associated with the above surgery and the anaesthesia required to facilitate the operation. Thereafter, unreservedly, and in my full senses, I have given my informed consent for administration of anaesthesia, conduct of the surgery or any other procedure (biopsy, venepuncture, transfusion or operation), as well as for additional molecular and genetic studies as required by the Hirschsprung study group that is found necessary during / following the operation/ recovery phase. I have been consented prior to blood sampling as well. I also agree to get my child's photographs taken by the study group.

I understand that the colonic biopsies and blood samples thus obtained may be stored and investigated with additional histological/histochemical and genetic/molecular studies at a later date. I have no objections for the same. This consent that I have given is purely voluntary. I understand that the results will be totally confidential and it remains with the investigators.

I give full consent to use my child's case details and photographs for research purposes, scientific publications and presentations in scientific forums by the above study group and guaranteed that our confidentiality would be maintained. I have been informed that if the techniques do not work successfully due to unforeseen causes, I would be called for a repeat sampling.

In the course of events, my child is confirmed not to have Hirschsprung disease or related disorders, I have no objection for the biopsy samples/blood samples/photographs of my child be used for research and scientific purposes.

For any issues, I have been asked to contact Dr Usha Kini, Professor of Pathology or Dr Kanishka Das, Professor and head of Paediatric surgery at 9448270384/ 9844055045 **Or IERB Convenor at 080-49466346/25634123.**

Signature / LTI of parent / legal guardian

Name _____Date _____

Signature of the Operating surgeon

Name _____Date _____

Signature of the Principal investigator

Name _____Date _____

Case Taken by:

APPENDIX- IV

Standard Operating Procedure for rapid H & E Staining Technique

Formula:

1. **Harris Haematoxylin:**

   In 100 ml distilled water in a large (3-4 litre) Erlenmeyer flask, dissolve 100 mg of aluminium or potash Alum by heating and shaking. Bring to 60^0C; add a solution of 5 gm of haematoxylin in 50 ml of absolute alcohol ethyl alcohol and bring rapidly to the boil. When it begins to boil, remove from flame and add 2.5 gm of mercuric oxide (red or yellow).

2. **Eosin Y:**

   Eosin Y - 5 gm

   Absolute alcohol - 100 ml

   Thymol may be added to prevent growth of fungus.

Technique:

- Dip the section in water
- Haematoxyline- 1 minute
- Wash in tap water
- Lithium carbonate- 1 dip
- Eosin- 2 dips
- Graded alcohol 2- 2 dips in each
- Xylene- 2 changes
- Mount with DPX.

Result:

Nuclei : Blue

Cytoplasm : Pale Pink

APPENDIX- V

Standard Operating Procedure for rapid Acetylcholinesterase Staining Technique

Preparation of Stock Solution:

Stock A

1. Preparation of acetate buffer- pH 6.0
    a) 0.2 M acetic acid pH 6.0
        - 0.3 ml of glacial acetic acid is mixed with 25 ml of distilled water
        - 2.1 ml of the above solution is taken
    b) 0.2 M sodium acetate
        - 0.82 gm of sodium acetate anhydrous is dissolved in 50 ml of distilled water
        - 47.9 ml of 0.2 M sodium acetate solution is used.
    c) Mix 2.1 ml of (a) with 47.9 ml of (b)
    d) Further add 50 ml of distilled water resulting in 100 ml of the acetate buffer.
2. Acetylthiocholine iodide 0.07 gm
3. Sodium citrate:
   M.W.- 294.1
   Weigh 0.732525 gm and dissolve in 25 ml distilled water
4. Copper sulphate
   M. W. – 159.6
   Weigh 0.2394 gm and dissolve in 50 ml of distilled water
5. Iso- octamethyl pyrophosphamide
   M.W. 342.4
   Weigh 0.0136 gm and dissolve in 10 ml of the distilled water
6. Distilled water- 5o ml.

For preparation of 15 tests

Solution A from Stock A- Blue coloured solution

- Acetylthiocholine iodide- 0.005 gm
- Acetate buffer- 97.5 ml
- Sodium citrate- 7.5 ml
- Copper sulphate- 15 ml
- Distilled water- 15 ml
- ISO OMPA- 3 ml

Mix in the above order and pipette out 9.2 ml into each tube.

To be kept in freezer compartment

Stock B

Potassium ferricyanide

M.W.- 329.2

Dissolve 0.0823gm in 50 ml of distilled water.

Solution B from stock B- Potassium ferricyanide.

Pipette 1 ml of potassium ferrricyanide into each of the small cuvettes.

To be kept in the freezer compartment

Note: 9.2 ml of stock A is mixed with 1 ml of stock B just before use.

Solution C- Rubeanic acid

Add 2.5 ml of absolute alcohol to Rubeanic acid just before use and shake it up to get good mix.

Solution D- 1.66 gm of sodium acetate

Add 10 ml of distilled water to sodium acetate just before use.

Staining Technique:

- Thaw solution A and solution B
- Mix solution B to A in slide box 1
- Immerse slide with cryostat cut sections in the solution mixture and keep the slide box 1 in the incubator at 37^0C for 20 minutes.

- One dip in water
- Transfer the slides to the slide box II containing solution C into which solution D is added.
- Keep for 10 minutes.
- One/ two dips in water.
- Dehydrate in various grades of alcohol
- Clear in xylene
- Mount with DPX.

APPENDIX- VI

Standard Operating Procedure of IHC staining technique

Specimen handeling:

1. Tissues are to be fixed in 10% neutral buffered formalin.
2. Cut paraffin embedded sections 4-5 microns thick are taken on APES coated slides.
3. Cut sections are to be fixed in the incubator at 60^0C overnight.

Reagents:

1. Hydrogen peroxide 30%
2. Methanol
3. Dibasic sodium hydrogen phosphate, anhydrous
4. Monobasic potassium phosphate, anhydrous
5. Sodium chloride
6. Sodium citrate
7. Citric acid
8. EDTA
9. Tris HcL
10. APES
11. Dako Real Envision detection system
12. DAB chromogen
13. Proteinase K
14. Primary antiobodies

Reagent Preparation:

APES (3- aminopropyltriethoic silane) coated slides for IHC

1. Label slides as APES with diamond marker
2. Immerse the slides in acetone for 10 minutes
3. Prepare APES solution by taking 10 ml of APES in 500 ml of acetone in a clean trough and mix well.
4. Remove the slides from acetone and immerse in the container of APES solution for 1-2 minutes
5. Remove slides and dry in the incubator at 60^0C.
6. Store in the freezer.

3%H_2O_2 in Methanol

1. Into clean glassware add 75 ml of methanol.
2. Add 25 ml of 30% H_2O_2 to the methanol and stir well

   Wear gloves while preparing this reagent.

Phosphate buffered saline (PBS) for wash 0.01 M, pH 7.2

Dissolve 1.48 gms of Na_2HPO_4, 0.43 gms of KH_2PO_4, 7.2 gms of NaCl in 1000 ml of Distilled water.

TBS (Tris Bufferd Saline) 1M pH7.4 (Stock)

Take 60.5 gms Tris in 400 ml of distilled water. Add conc. HCl approximately 37 ml. adjust pH 7.4 with HCl and then make the volume to 500 ml.

Working Tris Solution

Stock Tris- 100 ml
10x NaCl- 200 ml
Dist. H_2O- 2 Liters

Citrate buffer (pH6.0)

Stock A: 0.1 M Sodium nitrate (MW 294)
Dissolve 17.35 gms of sodium nitrate in 500 ml of Dist. H_2O
Stock B: 0.1 M Citric Acid (MW 210)
Dissolve 2.7 gms citric acid in 100 ml Dist. H_2O
Store in the fridge at 8^0C.

Citrate buffer working solution at pH6.0

Mix 38 ml of stock A, 12 ml of stock B and make up to 100 ml with Dist. H_2O

DAB Reagent Working

In the plastic vial add 20µl of DAB chromogen. Add 1 ml buffered substrate.

Tris EDTA buffer for antigen retrieval

Solutions and Reagents:

Tris- EDTA Buffer (10mM Tris, base, 1m M EDTA solution, 0.05% Tween 20, pH 9.0):

- Tris Base – 1.21 g

- EDTA - 0.37 g
- Dist. H_2O- 1000 ml

Mix to dissolve. pH is usually at 9.0 and then add 0.5 ml of Tween 20 and mix well. Store this solution at room temperature for 3 months or at4^0C for longer storage.

EDTA Buffer (0.1 M at pH 8) for antigen retrieval: Stock solution

Dissolve 37.2 gms of EDTA in 1 liter distilled Dist. Water
Adjust pH to 8 with HCl or NaOH
The solution is autoclaved after adjusting pH will last longer.

Working Solution:

Dilute 5 ml in 495 ml of De-ionized water (0.37 gms) and use.

Primary antibody dilution:

- Primary antibody should be reconstituted according to manufacturer's instructions if receive lyophilized
- Use PBS buffer to dilute primary antibodies.
- Once diluted, transfer antibody to plastic vial, labelled with antibody name, date made, dilution.

These antibodies are to be stored at 4^0C.

Staining Technique:

- Cut sections to be stained in APES coated slides
- Place racks containing slides in 60^0C for baking over night for processing the next day
- Deparaffinise the sections on slides in xylene with agitation - 15 minutes
- Wash in alcohol - 5 minutes
- Wash in distilled water
- Antigen retrieval
- Wash in buffer
- 3% Hydrogen peroxide - 10 minutes
- Wash in Buffer I - 5 minutes
- Wash in Buffer II - 5 minutes
- Primary antibody (E.g., CD117, Synaptohysin etc) - 1 hour

- Wash in Buffer I - 5 minutes
- Wash in Buffer II - 5 minutes
- Dako REAL™ EnVision™ - 30 minutes
- Wash in Buffer I - 5 minutes
- Wash in Buffer II - 5 minutes
- Chromogen DAB - 5- 10 minutes
- Stained with Hematoxylin (light) - 5-10 seconds
- Wash in water and dry well
- Mount with DPX and label the slides

Note:

1. Dako REAL™ EnVision™ Detection system, peroxidase/ DAB, Rabbit/Mouse kit is employed in two step procedure. The first step is incubation of the tissue with an optimally diluted primary rabbit or mouse antibody and the second step is incubation with Dako REAL™ EnVision™/HRP, Rabbit/Mouse (ENV) reagent of the kit. This reagent is a peroxidase-conjugated polymer, which also carries antibodies to rabbit and mouse immunoglobulins. This reaction is visualized by Dako REAL™ EnVision™ DAB + Chromogen, which is also included in the kit.
2. A positive control as recommended by the manufacturer is run with each antigen tested.
3. Antigen retrieval by enzyme method:
   Place the slide on a rack and cover the section with reconstituted trypsin/ proteinase K as recommended.
4. Antigen retrieval by pressure cooking:

Place the required buffer in a pressure cooker. When the buffer reaches boiling temperature, put the slides in a rack and close the pressure cooker. When the steam comes out put the whistle and keep for 5- 8 minutes. Put off the pressure cooker and let it cool to room temperature. Then proceed with IHC procedure.

APPENDIX- VII

Standard Operating Procedure for DNA Extraction from EDTA Blood sample using QIAMP DNA Mini Kit:

Requirements:

Instruments:

1. Thermomixer comfort- 1.5 ml Eppendorf.
2. Eppendorf Centrifuge 5810R
3. -20^{0}C freezer
4. BIO BEE^{TM} Cyclomixer- minispin.

Reagents:

1. QIAGEN Protease
2. Buffer AL
3. Buffer AW1
4. Buffer AW2

Reagents Preparation:

1. QIAGEN Protease stock solution (store at 2-8^{0}C or -20^{0}C)

Pipette 1.2 ml protease solvent (contains sodium azide as a preservative) into the vial containing lyophilized QIAGEN Protease, as indicated on the label. Dissolved QIAGEN protease is stable for up to 2 months when stored at 2-8ºC.

2. Buffer AL (store at room temperature, 15-25^{0}C) - Contains Chaotropic salt

Mix Buffer AL thoroughly by shaking before use. Buffer AL is stable for 1 year when stored at room temperature.

3. Buffer AW1 (store at room temperature, 15-25^{0}C) - Contains Chaotropic salt

Buffer AW1 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96-100%) as indicated on the bottle.

Buffer AW1 is stable for 1 year when stored at room temperature.

4. Buffer AW2 (store at room temperature, 15-25^{0}C) - Contains sodium azide as a preservative.

Buffer AW2 is supplied as a concentrate. Before using for the first time, add the appropriate amount of ethanol (96-100%) to Buffer AW2 concentrate as indicated on the bottle.
Buffer AW2 is stable for 1 year when stored closed at room temperature.

Procedure:

1. Pipet 20 µl QIAGEN Protease (or proteinase K) into the bottom of a 1.5 ml microcentrifuge tube.
2. Add 200 µl sample to the microcentrifuge tube.
3. Add 200 µl Buffer AL to the sample. Mix by pulse- vortexing for 15 seconds.
4. Incubate at 56^{0}C for 10 minutes.

DNA yield reaches maximum after lysis for 10 mins at 56^{0}C.

5. Briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

6. Add 200 µl ethanol (96-100%) to the sample, and mix again by pulse- vortexing for 15 s. After mixing, briefly centrifuge the 1.5 ml microcentrifuge tube to remove drops from the inside of the lid.

7. Carefully apply the mixture from step 6 to the QIAamp Mini spin column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g(8000 rpm) for 1 min. Place the QIAamp Mini spin column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

Close each spin column in order to avoid aerosol formation during centrifugation.

8. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW1 without wetting the rim. Close the cap and centrifuge at 6000 x g(8000 rpm) for 1 min. Place the QIAamp Mini spin column in a 2 ml collection tube (provided), and discard the collection tube containing the filtrate.

9. Carefully open the QIAamp Mini spin column and add 500 µl Buffer AW2 without wetting the rim. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

10. Place the QIAamp Mini spin column in a new 2 ml collection tube (not provided) and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

This step helps to eliminate the chance of possible Buffer AW2 carryover.

11. Place the QIAamp Mini spin column in a clean 1.5 ml microcentrifuge tube (not provided) and discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column and add 200 µl Buffer AE or distilled water. Incubate at room temperature (15-25^{0}C) for 1 min, and then centrifuge at 8,000 rpm for 1 min.

Result:

The extracted DNA by this method has run in the gel as a part of standardization procedure and optimum band has been observed.

APPENDIX- VIII

60th PPS Annual Meeting (Jointly with the SPP)

2014

Unique ID:	**586**
Abstract Title:	**Improvised double-embedding technique of minute biopsies in Hirschsprung disease: A mega boon to histopathology laboratory**
Abstract Authors:	1. Lokendra Yadav, Research scholar Department of Pathology, St. John's Medical College, Bangalore, India. 2. Sarega Thomas (M.Sc) Amity University, Noida, India. 3. Usha Kini, Professor of Pathology, St. John's Medical College, Bangalore, India.
Abstract Text:	**Introduction:** Optimal orientation of rectal mucosal/seromuscular biopsies is essential to visualize neural plexuses for a definite diagnosis of Hirschsprung disease (HD). The problem of orientation of such biopsies when minute gets compounded when they are from neonates and mandates exhaustive strip cuts, thus delaying diagnosis. **Aim:** A modified agar-paraffin technique is aimed to make tissue embedding efficient and user-friendly by inking fresh/ fixed mapping biopsies followed by embedding in agar after orientation and re-embedding the agar block in paraffin wax after tissue processing. **Material & Methods:** One hundred and fifty two mucosal/seromuscular biopsies from suspect HD cases were processed by this improvised double embedding method with colouring inks and sections analysed microscopically. **Results:** The tissue in agar paraffin blocks were well processed, firm, held secure, easy to cut with serial sections of desired thickness and spread without folds. The inks remained permanent on the tissues in the block and on microscopic sections, thus helping in easy identification of tissues. Agar did not interfere with histological/histochemical stains or with AChE enzyme histochemistry/ immunohistochemistry. Differential inking of mapping biopsies from the same patient and pooling them onto a block reduced the number of tissue blocks, the worktime and reagents markedly. **Conclusion:** The improvised agar-paraffin embedding technique for mucosal/seromuscular biopsies for HD is a simple reliable user friendly method that helps to obtain perfect orientation, fast turnaround time for both frozen and formalin fixed biopsy and cost-effectiveness by economizing on the number of paraffin blocks, chemical reagents and manpower.
Abstract Type:	oral
Presentation:	08:45hrs on Friday 5th September 2014
Abstract Sponsor:	Professor Vijay V Joshi
Corresponding Author:	Lokendra Yadav

APPENDIX- IX

60th PPS Annual Meeting (Jointly with the SPP)

2014

Unique ID: 594

Abstract Title: **Calretinin immunohistochemistry Vs rapid Acetylcholinesterase histochemistry in Hirschsprung disease: Who wins and when?**

Abstract Authors:
Lokendra Yadav, Dept of Pathology, St. Johns Medical College, Bangalore, India.

Usha Kini, Dept. of Pathology, St. Johns Medical College, Bangalore, India.

Kanishka Das, Dept. of Paediatric Surgery, St.Johns Medical College, Bangalore, India.

Suravi Mohanty, Dept. of Pathology, St. Johns Medical College, Bangalore, India.

Divya Puttegowda, Dept. of Pathology, St. Johns Medical College, Bangalore, India.

Abstract Text:
Introduction: Acetylcholinesterase (AChE) histochemistry on rectal mucosal biopsies considered the goldstandard for diagnosis of Hirschsprung disease (HD) is not widely employed as it requires special tissue handling and pathologist expertise. Calretinin immunohistochemistry (IHC) has been reported to be comparable to AChE staining with loss of calretinin expression correlating with aganglionosis.

Aim: To evaluate calretinin IHC as a primary diagnostic tool in comparison to improvised rapid AChE technique in the diagnosis of HD.

Material and Methods: Seventy four rectal biopsies (18 fresh frozen, 56 formalin fixed) from 51 cases of suspect HD were evaluated with H&E, AChE (a 40 mins staining technique) and Calretinin using a protocol. Known ganglionated and aganglionated segment biopsies served as positive and negative controls. Two pathologists blinded to the clinical details evaluated each biopsy and their observations were statistically analyzed to assess the correlation between Calretinin and AChE and the inter observer agreement.

Results: AChE highlighted the dark greenish black hypertrophic nerves in HD and calretinin defined the delicate mucosal fibres in nonHD. The study confirmed HD in 26 and non HD in 25 cases. The results of calretinin were comparable to AChE with a statistically significant measure of agreement (kappa 0.973) between the two. One false positive was noted with calretinin.

Conclusion: Calretinin is a reliable single immune marker for ruling out HD by its specific positive mucosal staining of formalin fixed rectal biopsy in non HD. However, the improvised AChE histochemistry remains indispensable to confirm HD on fresh biopsies, thus facilitating surgical decisions based on conclusive intra-operative diagnosis

Abstract Type: oral

Presentation: 09:45hrs on Friday 5th September 2014

Abstract Sponsor: Dr.Vijay V Joshi

Corresponding Author: Divya Puttegowda

APPENDIX- X

OP/33
Expression of *p*53, Ki-67 and proliferating cell nuclear antigen (PCNA) in uterine smooth muscle tumors
Poonam Rani, Vinay Kamal, Nita Khurana, Ashok Kumar
Maulana Azad Medical College and associated Lok Nayak Hospital, New Delhi, India

Objective: To correlate them clinicopathologically. **Materials and Methods:** Eighty seven cases of uterine smooth muscle tumors were studied, which included 4 malignant and 83 benign tumors. Detailed clinical history was recorded in all cases. Gross, microscopic and immuno-histochemical features were studied. Statistical correlation was done using Chi Square test and Fisher's exact test. **Results:** *p*53 and Ki-67 expression were found to be significantly higher in leiomyosarcomas (LMS) as compared to leiomyomas and its variants ($P < 0.001$). Hence high *p*53 and Ki-67 expression in uterine smooth muscle tumors is an indicator of malignancy. There was significantly increased PCNA expression in leiomyosarcomas than in uterine leiomyomas ($P = 0.001$). Co-expression of Ki-67 and *p*53 correlates well with LMS, while negativity for both these markers correlates well with leiomyoma and its variants. Co-expression of *p*53, Ki-67 and PCNA index >15% correlate well with LMS. **Conclusion:** Although benign and malignant uterine smooth muscle tumors can easily be differentiated morphologically but few variants of leiomyomas and borderline tumors pose diagnostic difficulties. Immunohistochemistry can help in solving this diagnostic dilemma. P53 and proliferative markers such as Ki-67 and PCNA are expressed more in malignant tumors. However, Ki-67 proved to be better than PCNA in differentiating benign uterine smooth muscle tumors from malignant ones. Hence a panel of markers including *p*53, Ki-67 and PCNA can be used in diagnosing problematic uterine smooth muscle tumors.

OP/34
Duodenal Carcinoids Revisited: A Clinicopathological Study of 35 Cases
Christol B. Moras, Anuradha Ananthamurthy, Marjorie Correa, Mallikarjun Patil
Departments of Pathology and Gastroenterology, St. John's Medical College, Bangalore, Karnataka, India

Introduction: Duodenal carcinoids (well differentiated neuroendocrine tumors) are believed to be rare neoplasms accounting for only 2-3% of all gastrointestinal (GIT) carcinoids in the published literature. However carcinoids of the duodenum accounted for nearly 61% of all carcinoids of the GIT diagnosed in our institution over a 6 year period, making it the most common location of occurrence. **Aims and Objectives:** To study the clinical and pathological profiles of cases of duodenal carcinoids presenting to our institution. **Materials and Methods:** A total of 35 patients with duodenal carcinoids who presented to our institution from 2006 till present were included in the study. The clinical data were obtained from the case files and histopathology slides were reviewed. **Results:** Of the 35 cases of duodenal carcinoids, 24 were males and 11, females. T he patients ranged from 19 years of age to 72 years. The majority (71%) presented as single nodules. One patient also had a concurrent carcinoid of the stomach. The size of the tumors ranged from 0.5 cm to 4.5 cm. All tumors except one were limited to the mucosa and submucosa; one tumor showed infiltration up to the serosa. One patient had a concurrent carcinoma of the stomach and one had a carcinoma of the esophagus. None had symptoms attributable to the carcinoid syndrome. **Conclusions:** This study, one of the largest case series from India, highlights the profile of duodenal carcinoids in our patients. Whether H pylori induced atrophic gastritis plays a role in the pathogenesis of these tumors is a matter worthy of consideration.

OP/35
Xanthogranulomatous Lesions. A Close Mimicker of Malignancy: Review of Literature with Special Emphasis on IHC and Special Stains
Irfan Ansari, Geetha Prakash, V. Bhanumathi, K. Rajeswari, V. Eswari, Shuaeb Bhat
Department of Pathology, Meenakshi Medical College and RI, Kanchipuram, Tamilnadu, India

Introduction: Xanthogranulomatous lesions (XGL) are uncommon pathological diagnosis, but a well-recognized disease entity of the kidney and gallbladder. However, XGL of other organs are rarely reported. XGL causes destruction and effacement of the normal structures of the involved organ and could be misinterpreted as a locally invasive cancer. We tried to define the precise histopathologic features of the XGL from various organs available in our archives. **Materials and Methods:** This study included 18 cases of XGL of various organs which include gall bladder, kidney, testes, ovary, prostate, breast, bone, stomach and subcutaneous soft tissue. Almost in all cases a preoperative diagnosis of carcinoma was given. Special stains (acid-fast, GMS, PAS, AB and mucicarmine), IHC (CD 68, cytokeratin, *S*-100, CD117 and vimentin) were applied. **Results:** All cases showed XGL with foamy histiocytes, lymphocytes and foreign body-type giant cells, which were positive for CD68 and negative for CD117, *S*-100 and cytokeratin. **Discussion:** The pathogenesis of XGL is not well understood. Various mechanisms have been suggested including chronic recurrent infection, gallstone or kidney stone, defective lipid transport, infectious agents and immunological factors. *Escherichia coli* is one of the causes of XGL in the kidney and gallbladder. **Conclusion:** XGL causes destruction and effacement of the normal structures of the affected organ and are often misdiagnosed as neoplasm, hence an extensive Histopathologic review of the tissue and battery of other investigations like special stains and IHC should be taken under consideration before excluding a diagnosis of neoplasm.

OP/36
Evaluation of VEGF expression in colorectal adenocarcinoma as a prognostic marker
Pavitra Kondety, K. Durga, P. Nalini Kumari
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Introduction: The formation of new blood capillaries from existing vessels is known as angiogenesis. It is an important mechanism for supplying nutrients to cells that are distant from existing blood vessels. Cancer progression is dependent on the development of a rich vascular network that supplies nutrients to the growing tumor. Vascular endothelial growth factor is one of the potent angiogenic factors. VEGF (vascular endothelial growth factor) system is a part of the platelet derived growth factor gene family. VEGF-A is a very potent angiogenic growth factor that interacts with specific receptors VEGFR-1 and VEGFR-2. VEGF expression has been successfully quantified via immunohistochemistry in colorectal cancers. **Aims and Objectives:** To investigate the expression of VEGF in colorectal adenocarcinoma and to evaluate its prognostic significance by comparing with known prognostic factors like age at presentation, size of tumor, histological type, no of lymph nodes showing metastasis etc. **Materials and Methods:** A retrospective study has been conducted from June 2010 to May 2012. A total of 24 cases of colorectal adenocarcinoma were collected and immunohistochemistry was performed on them. **Results:** Our study showed that VEGF expression was positive in tumors of greater size, tumors with lymph nodal metastasis at the time of presentation and patients of older age group. Expression of VEGF correlated with poor prognosis.

OP/37
Role of syanptophysin in ynfracte circumferential full thickness (doughnut) bowel biopsy in Hirschsprung's disease
Usha Kini, Babu M. K., Lokendra Yadav, Suravi Mohanty, Divya P., Saleem K. M., Kanishka Das
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Objectives: Retained segment of aganglionic bowel may cause persistent symptoms in Hirschspung disease after colostomy/pull through procedures. Currently we practice a combination of frozen H and E and acetylcholinesterase (AchE) histochemistry for the intraoperative leveling. As AchE activity is not reliable and may not be helpful in biopsies proximal to the sigmoid colon, IHC for synaptophysin (SY) may be helpful in these situations. **Materials and Methods:** This is a

prospective cross sectional study from a referral centre for HD in India over a 2 year period. 38 fresh doughnuts (circumferential full thickness) bowel biopsy from the suspected distal normally innervated ganglionic bowel in patients with HD were studied along with 20 doughnuts from resected colorectal specimens of non HD cases (controls). Frozen sections were cut and concurrently stained with rapid hematoxylin and eosin and 40 minute modified rapid AchE staining technique. IHC staining for synaptophysin using clone SY38 MAB5258-20UG was studied with a detailed protocol. Results: Of the 38 cases of HD, 28/28 recto-sigmoid type showed normal circumferential innervations with transition zone in sigmoid while 10 showed abnormal innervation in long segment HD (7) and total colonic aganglionosis (3) wherein the suspected transition zone was in transverse colon and affecting the entire circumference (8), three quadrants (2), two quadrants (1) and one quadrant (6) of the doughnut. The doughnuts with normal innervation pattern (H and E, AchE) showed intense SY activity in both the muscle layers, ganglion cells in the neural plexuses as well as in the mucosa. The doughnuts with abnormal ÿnfracted ns in all four quadrants showed no SY structures in both muscle layers of muscularis and no ganglion cells. The mucosa showed no SY fibers and fewer neuroendocrine cells. The transition zone with abnormal innervations involving one to three quarters showed fewer SY fibers in the muscularis with occasional ganglion cells highlighted by SY. The hypertrophic nerve bundles seen were SY negative unlike SY positive serosal nerve fibers seen in the controls. Conclusion: SY IHC is an indirect labeling method with a high detection rate for intestinal ganglion cells; it reflects their function by demonstrating synapses in the muscle fibers. The pattern and intensity of SY-positive synapses and their distribution in circular and longitudinal muscles can identify segments with abnormal ÿnfractedÿns and hence reflect functional disturbances of colonic motility. Thus, they can be used as tissue marker of normal ÿnfractedÿns for leveling in the management of HD.

OP/38

Leucoerythroblastosis: The versatile phenomenon!!

Catherine Matthew, M.C.S Savithri
Department of Pathology, Amala Institute of Medical Sciences and Cancer Center, Thrissur, Kerala, India

Objectives: To find the spectrum of causes of leucoerythroblastosis and to correlate the causes with peripheral smear morphological findings. Materials and Methods: A retrospective analytical study was conducted during the period of June 2010 to June 2012. All peripheral smears with the impression of leuco-erythroblastosis were selected and final diagnoses of these cases were recorded. The morphological changes in RBCs, WBCs and Platelets were analyzed and correlated with the final diagnosis of the patients Results: A total number of cases were 41 of which 22 (53.65%) were malignant causes and 19 (46.34%) were non malignant causes. Of the 22, 15 were hematological malignancies and 7 were non hematological malignancies. Non malignant etiologies showed a wide spectrum ranging from Dengue hemorrhagic fever to myocardial infarction. Most patients were in the fifth and sixth decade (29.26%) of life. There was no statistically significant difference in the sex wise distribution of leucoerythroblastosis. Peripheral smear morphological parameters like Tear drop cells, acanthocytes, blast cells, shift to left and association of thrombocytopenia were assessed that pointed to the underlying cause. Conclusions: An impression of leucoerythroblastosis should alert the clinician to the possibility of an underlying malignancy but not always. Therefore, an awareness of the varied non-malignant causes of leucoeythroblastosis avoids unnecessary painful investigations for the patient.

OP/39

Hemoglobin E syndromes: A tertiary care centre experience

Indu M., Shanthala Devi A.
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Introduction and Objectives: Hemoglobin E is an important hemoglobin variant seen commonly in South East Asia and cases have been reported from Northeast India. The hemoglobin E syndromes encompass homozygous, heterozygous and compound heterozygous states. Accordingly the clinical picture can range from asymptomatic presentations to a transfusion dependent state. The aim of this study is to describe and correlate the ÿnfrac-hematological features of Hb E syndromes. Materials and Methods: Cases of Hb E syndromes diagnosed in St Johns hospital during the study period were included in the study and the case charts were reviewed for ÿnfrac-hematological details. Results: Seventy nine cases (41 males and 38 females) of Hemoglobin E syndromes were diagnosed during a period of 4 years from 2007 to 2011. 51.9% (n = 41) were Hb E trait, 31.6 % (n = 25) were homozygous Hb E E disease and 16.5% (n = 13) were Hb E-beta thalassemia cases. The age of the patients ranged from 1 year to 61 years (n = 29). 79.75% of the cases were from Northeast India. The clinical features included pallor, icterus and hepatosplenomegaly. The Hb% ranged from 4.1 to 16.3 g%. The peripheral smear showed microcytic/normocytic hypochromic blood picture with target cells. The clinical features were more severe in compound heterozygotes of Hb E-Beta thalassemia and homozygotes and this showed a significant positive correlation. (Spearman's correlation $P < 0.005$). Conclusion: Hb E syndromes should be considered in the differential diagnosis of hemolytic anemias and the severity of the clinical symptoms correlates with the genotype.

OP/40

Evaluation of Fine needle aspiration cytology of lymph nodes in Tertiary care Hospital, Mumbai

Jayalakshmi V., N. P. Chikhale, Aradhana Mishra, Susan Cherian, Chaturvedi, Richa Patel
Bhabha Atomic Research Centre Hospital, Mumbai, India

Objectives: To evaluate the results of fine needle aspiration cytology (FNAC) of lymph nodes in our institution and compare with histopathology wherever possible. Materials and Methods: FNAC from 500 patients of lymphadenopathy were studied over approximately four years. Results: Age of patients ranged between 1-95 years and F:M ratio was 1:1.36. Most frequently involved nodes were cervical (52.8%) followed by supraclavicular (15.2%), submandibular (10.4%), axillary (7.2%) and inguinal (4.6%). Multiple nodes were involved in 2.6% patients. FNAC was diagnostic in 79.4% cases. Overall prevalence of various lesions was reactive hyperplasia in 28.4%, Tuberculosis in 17.4%, granulomatous lymphadenitis in 16%, acute suppurative lymphadenitis in 5.8%, metastatic in 5%, chronic lymphadenitis in 3.4%, lymphoma in 1.46%. Males showed preponderance of metastases, Tuberculosis showed female preponderance while cases of lymphoma were equal in both. Among 32 cases of malignancy, 78.12% had metastases and 21.87% had lymphoma. AFB was positive in 31.03% cases of tuberculosis. Of the 32 cases of granulomatous lymphadenitis subjected to surgical excision, 26 (81.25%) were diagnosed as tuberculosis and a single case was Hodgkin disease. 26 cases of reactive lymphoid hyperplasia where histopathologic examination was done, revealed tuberculosis in 11 (42.3%), toxoplasmosis in 2 patients and Kikuchi Fujimoto disease and Hodgkin disease in one each. Conclusion: FNAC is useful and reliable in diagnosing neoplastic and non-neoplastic lesions of lymph nodes. It is an excellent first line method for investigating the nature of the lesions and is economical and convenient alternative to open biopsy.

OP/41

An analysis of lymph node yield after neoadjuvant chemoradiation in rectal cancer: Does size matter?

Mousumi Kar, Susy Kurian, Divya Midha, Indu Arun, Manas Roy, Sudeep Banerjee, Amit Dwary, Indranil Mallick, Paromita Roy
Departments of Pathology, Surgical Oncology, Medical Oncology, Radiation Oncology, Tata Medical Center, Kolkata, India

Aims: Neoadjuvant chemoradiation (NACRT) is the current standard of care before surgery for rectal adenocarcinoma. Lymph node yield is an important determinant of prognosis, but it is widely documented that lymph node yield is substantially lower after NACRT, with mean yields

APPENDIX-XI

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Dr. Usha Kini
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Dear Dr Kini

We are pleased to inform you that your manuscript entitled

Role of synaptophysin in the Intra-Operative Assessment of Quadrantic Inner Proximal Doughnut in Hirschsprung Disease

has been accepted for publication in The National Medical Journal of India. It will near future.

Thanking you,

Yours sincerely,

(Peush Sahni)