A Dissertation on

STUDY OF CALRETININ IMMUNOHISTOCHEMISTRY STAINING PATTERN AND ITS HISTOPATHOLOGICAL CORRELATION IN DIAGNOSIS OF HIRSCHSPRUNG'S DISEASE

COIMBATORE MEDICAL COLLEGE

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Dissertation Submitted to

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With partial fulfilment of the regulations

for the award of the degree of

M.D – PATHOLOGY (Branch III)

MAY 2019

DECLARATION

I solemnly declare that this dissertation entitled ***"STUDY OF CALRETININ IMMUNOHISTOCHEMISTRY STAINING PATTERN AND ITS HISTOPATHOLOGICAL CORRELATION IN DIAGNOSIS OF HIRSCHSPRUNG'S DISEASE"*** was done by me in the Department of Pathology, Coimbatore Medical College, Coimbatore from August 2016 to March 2018 during the academic years 2016-2019 under the guidance and supervision of **Dr.C.Lalitha, M.D.**, Professor and Head, Department of pathology, Coimbatore Medical College, Coimbatore.

This dissertation is submitted to **The Tamilnadu Dr.M.G.R. Medical University,** Chennai towards the partial fulfilment of the requirement for the award of M.D., Degree (Branch III) in Pathology. I have not submitted this dissertation on any previous occasion to any University for the award of any Degree.

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This is to certify that the dissertation entitled ***"STUDY OF CALRETININ IMMUNOHISTOCHEMISTRY STAINING PATTERN AND ITS HISTOPATHOLOGICAL CORRELATION IN DIAGNOSIS OF HIRSCHSPRUNG'S DISEASE"*** is a record of bonafide work done by **Dr**.**R.ARTHIPRIYADHARSINI**, a Post Graduate student in the Department of Pathology, Coimbatore Medical College and Hospital, Coimbatore under the guidance and supervision of **Dr.C.LALITHA, M.D.**, Professor and Head, Department of pathology, Coimbatore Medical College and Hospital, Coimbatore in partial fulfilment of the regulations of The Tamilnadu Dr.M.G.R. Medical University, Chennai towards the award of M.D., Degree in Pathology (Branch III).

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have 95% accuracy in HD diagnosis. When additional immunohistochemical studies were carried out, the correct diagnosis was shown to have very high sensitivity, up to 99.7% [7] . A number of ancillary methods like enzyme histochemistry using Acetylcholinesterase, NADPH-diaphorase etc, and immunohistochemistry (IHC) stains like neuron specific enolase (NSE), Calretinin, c-kit, S-100 etc. have been studied to overcome these limitations and to improve the diagnostic accuracy in specimens. Acetylcholinesterase staining can be performed only in frozen sections and requires additional specimen for H&E. Among IHC staining studies have shown a better diagnostic accuracy with Calretinin.

So this study compares the diagnostic sensitivity of H&E and Calretinin IHC in suspected cases of HD and the staining pattern of ganglion cells in various layers of bowel tissue. Interobserver concordance in diagnosis is also evaluated.

AIMS & OBJECTIVES 1. To examine the sensitivity of Calretinin immunohistochemistry compared to conventional Hematoxylin & Eosin (H&E) sections in the diagnosis of Hirschsprung disease. 2. To compare the interobserver concordance in H&E and Calretinin immunohistochemistry. 3. To assess the staining pattern of ganglion cells and nerve fibers in both ganglionic and aganglionic segment of bowel.

REVIEW OF LITERATURE Hirschsprung's disease (HD) is a common cause of neonatal intestinal obstruction. Being a congenital disorder has an incidence of 1 in 5000 live births. But it ranges from 1 in 2,000 to 1 in 12,000 live births over the world. California Birth Defects Monitoring Program had found the highest incidence in Asian populations about 2.8 in 10,000 live births [3] . It has been recognized that males are more commonly affected than females with a male: female ratio of 4:1 [9, 10, 11] . The neonates usually presents with

CERTIFICATE - II

This is to certify that this dissertation work titled **"STUDY OF CALRETININ IMMUNOHISTOCHEMISTRY STAINING PATTERN AND ITS HISTOPATHOLOGICAL CORRELATION IN DIAGNOSIS OF HIRSCHSPRUNG'S DISEASE"** of the candidate ***Dr.R.ARTHIPRIYADHARSINI***, with registration number 201613251 for the award of MD degree in the branch of PATHOLOGY. I personally verified the urkund.com website for the purpose of plagiarism check. I found that the uploaded thesis file contains from introduction to conclusion pages and result shows **Twelve percentage (12%)** of plagiarism in the dissertation.

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Dr.R.ARTHIPRIYADHARSINI

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ABBREVIATIONS

- AChE - Acetyl choline esterase
- DAB - Diaminobenzidine
- DPX - Destrene, Phthalate, Xylene
- ENS - Enteric Nervous System
- HD - Hirschprung's disease
- H & E - Hematoxylin and Eosin
- ICC - Interstitial cells of Cajal
- IHC - Immunohistochemistry
- LP - Lamina Propria
- LHD - Long segment Hirschsprung's disease
- MM - Muscularis Mucosa
- MP - Muscularis propria
- NADPH - Nicotinamide adenine dinucleotide
- NC - Neural crest
- NPV - Negative predictive value
- NHD - Non Hirschsprung's Disease
- PPV - Positive predictive value
- SHD - Short segment Hirschsprung's disease
- SM - Sub mucosa
- TBS - Tris buffer saline
- TCA - Total colonic aganglionosis

PROFORMA I (for H&E)

Department Of Pathology

Coimbatore Medical College, Coimbatore.

Name: IP no:

Age: HPE no:

Sex: Ward:

Type of specimen: Biopsy/ Resected specimen

Extent of Disease: SHD/LHD/TCA

OBSERVER **One / Two**

GANGLIONIC SEGMENT:

- **Ganglion cells:**
  Submucosa Present/ absent/ suspicious
  Muscularis propria Present/ absent/ suspicious
- **Hypertrophic nerve fibres:**
  Submucosa Present/ absent/ suspicious
  Muscularis propria Present/ absent/ suspicious

AGANGLIONIC SEGMENT:

- **Ganglion cells:**
  Submucosa Present/ absent/ suspicious
  Muscularis propria Present/ absent/ suspicious
- **Hypertrophic nerve fibres:**
  Submucosa Present/ absent/ suspicious
  Muscularis propria Present/ absent/ suspicious

Final diagnosis: HD/ NHD/ Suspicious

PROFORMA II (for Calretinin IHC)

Department Of Pathology

Coimbatore Medical College, Coimbatore.

Name: IP no:

Age: HPE no:

Sex: Ward:

Type of specimen: Biopsy/ Resected specimen

Extent of Disease: SHD/LHD/TCA

OBSERVER **One / Two**

GANGLIONIC SEGMENT:

- **Ganglion cells:**
  Submucosa Present/ absent/ suspicious
  Muscularis propria Present/ absent/ suspicious
  Immunoreactivity index:
- **Intrinsic nerve fibres:**
  Lamina propria Present/ absent/ suspicious
  Muscularis mucosa Present/ absent/ suspicious
  Submucosa Present/ absent/ suspicious
  Muscularis propria Present/ absent/ suspicious

AGANGLIONIC SEGMENT:

- **Ganglion cells:**
  Submucosa Present/ absent/ suspicious
  Muscularis propria Present/ absent/ suspicious
- **Intrinsic nerve fibres:**
  Lamina propria Present/ absent/ suspicious
  Muscularis mucosa Present/ absent/ suspicious
  Submucosa Present/ absent/ suspicious
  Muscularis propria Present/ absent/ suspicious

Final diagnosis: HD/ NHD/ Suspicious

INTRODUCTION

Hirschsprung's disease (HD) or aganglionic megacolon is a congenital anomaly of innervation of the lower intestine, usually limited to the colon, resulting in partial or total functional obstruction [1]. The estimated incidence is 1 in 5000 live births [2, 3]. HD is caused by congenital absence of the Meissner's and Auerbach's autonomic plexus (aganglionosis) in the intestinal wall. Disease is usually limited to the distal colon (75% of cases) but can involve the entire colon or even the entire large and small bowels; the denervated area is always contiguous.

The etiology of the aganglionosis is thought to be the failure of migration of neuroblasts from the neural crest. There is a significant genetic component to this disorder at least 12 different genetic mutations and many syndromes are associated with HD.

HD can be suspected clinically by the presenting symptoms, the typical appearance of the bowel radiology after contrast enema and by measuring recto-anal pressure using rectal manometry. Above all, gold standard is the demonstration of the absence of ganglion cells in the histopathological sections of rectal biopsy [4]. This helps in arriving at the definite diagnosis of HD.

Hallmark histological finding is aganglionosis and presence of numerous hypertrophic cholinergic nerve fibers [5, 6]. So the suspected child usually undergoes suction biopsy or full-thickness biopsy for confirmation. Specimen adequacy is very essential for diagnosis of HD in routine Hematoxylin & Eosin (H&E) sections. The diagnosis of HD can be difficult on H&E alone and requires good experience, principally for pathologists who infrequently encounter the disease. H&E has limitations in term of examining those submucosal areas where ganglion cells are small and irregularly distributed, also difficulty in identifying immature ganglion cells of neonates. H&E has shown to have 95% accuracy in HD diagnosis. When additional immunohistochemical studies were carried out, the correct diagnosis was shown to have very high sensitivity, up to 99.7% [7]. A number of ancillary methods like enzyme histochemistry using Acetylcholinesterase, NADPH-diaphorase etc, and immunohistochemistry (IHC) stains like neuron specific enolase (NSE), Calretinin, c-kit, S-100 etc. have been studied to overcome these limitations and to improve the diagnostic accuracy in specimens. Acetylcholinesterase staining can be performed only in frozen sections and requires additional specimen for H&E. Among IHC staining studies have shown a better diagnostic accuracy with Calretinin.

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1. To examine the sensitivity of Calretinin immunohistochemistry compared to conventional Hematoxylin & Eosin (H&E) sections in the diagnosis of Hirschsprung disease.

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REVIEW OF LITERATURE

Hirschsprung's disease (HD) is a common cause of neonatal intestinal obstruction. Being a congenital disorder has an incidence of 1 in 5000 live births. But it ranges from 1 in 2,000 to 1 in 12,000 live births over the world. California Birth Defects Monitoring Program had found the highest incidence in Asian populations about 2.8 in 10,000 live births [8]. It has been recognized that males are more commonly affected than females with a male: female ratio of 4:1 [9, 10, 11]. The neonates usually presents with distended abdomen, feeding intolerance with bilious aspirates or bilious vomiting and classically with 'delayed passage of meconium'. It has been said that over 90% of HD infants fail to pass meconium in the first 24 hours of life [12]. Some children do not become obstructed in the neonatal period and present later in infancy or in adulthood with severe constipation, chronic abdominal distension and failure to thrive [13, 14]. After a careful history and physical examination, the diagnostic steps may include radiographic studies, anorectal manometry and a rectal biopsy. Of these histopathological examination is confirmatory.

HIRSCHSPRUNG'S DISEASE: A HISTORICAL PERSPECTIVE

Although this condition was initially described by Fredericus Ruysch in 1691, Harald Hirschsprung presented the first concise description of congenital megacolon in 1886 in his treatise "Constipation in newborns due to dilatation and hypertrophy of the colon" [15]. Since then many authors have tried to propose specific pathological feature and successful treatment for this condition. In 1920, Dalla Valla in their study noted aganglionosis in the sigmoid colon of the siblings [16] and in 1940 Whitehouse and Kernohan demonstrated that the aganglionosis within the distal colon or rectum was the cause of the functional obstruction [17] but still the origin of the innervation anomaly remained unclear. Bolande in the year 1974 used the term neurocristopathy to denote syndromes or tumours of neural crest cell origin [18]. The pull-through operational procedure was first done by Bill and Swenson in the year 1948. Soave and Duhamel developed two and three staged operations for this disease. The rectal mucosal biopsy technique was developed by Noblett in the year 1969 and this has revolutionised the diagnosis of Hirschsprung's disease [19]. So, along with his co-workers described the procedure for pull-through and single staged surgery in 1980.

ETIOPATHOGENESIS:

The basic pathophysiological feature in Hirschsprung's disease (HD) is a functional obstruction caused by a narrowed distal aganglionic colonic segment that prevents the propagation of peristaltic waves. Failure of neural crest cell migration, genes, cholinergic hyper-innervation, distributional abnormalities of neurofibrils, disturbance with nitric oxide synthetase (NOS) and cajal cell abnormalities all seemed to be involved in the pathogenesis of Hirschsprung's disease. Despite extensive research, the complex pathophysiology of HD is not fully understood. There is no clear explanation for the occurrence of spastic or tonically contracted aganglionic segment of bowel.

The **Enteric Nervous System** (ENS) is the largest and the most complex division of the peripheral nervous system plays a crucial role in normal gastrointestinal motility. Therefore insights into the embryonic origin and development of the ENS are relevant for the understanding of the pathophysiology of HD. The ENS contains more neurons than the spinal cord and is capable of mediating reflex activity in the absence of central nervous system. The ENS is composed of intrinsic neurons, the cell bodies and processes that are located inside the bowel wall and extrinsic nerve fibres that project into the gut from autonomic and sensory ganglia according to Furness JB [20] Although extrinsic

innervation modulates the activity of intrinsic neurons, the intrinsic neurons are necessary for the complex reflex pathways that ensure peristaltic activity. Nerves in both myenteric and submucosal nerve plexuses are a mixture of intrinsic and extrinsic fibres and specialized enteric glial cells. They project into muscularis propria, muscularis mucosa, and lamina propria according to Raj P. Kapur [21].The sympathetic and parasympathetic arms of the peripheral nervous system and the neurons of the ENS is derived primarily from cells of the vagal segment of the *neural crest* (NC)[22], a part of ectoderm. Problems in NC development are the basis of many human syndromes and birth defects known collectively as *Neurocristopathies*.

Neural crest cells are pluripotent stem cells, the epithelio-mesenchymal transformation allows NC cells to migrate along pathways of defined routes to various tissues, where they stop moving and differentiate into various cell types. In the human fetus, neural crest-derived neuroblasts first appear in the developing esophagus at 5 weeks of gestation, and they migrate down in a craniocaudal direction to the small intestine by the 7th week and reaches colon during the 12th week of gestation [23, 24]. The NC cells first form 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. In addition, after the craniocaudal migration has ended, the submucous plexus is formed by the neuroblasts, which migrate from the myenteric plexus across the circular muscle layer and into the submucosa; Normal ganglion cell distribution is present at 24 weeks of gestation in humans. These ganglia continue to mature on into childhood.

HD results when the normal migration of neural crest cells fails to reach the rectum or sometimes normal cell migration may occur but neuroblasts may be subject to apoptosis or there may be failure of proliferation, or improper differentiation within the affected distal intestinal segment. Fibronectin, laminin, neural cell adhesion molecule (NCAM), and neurotrophic factors present in the intestinal stroma are necessary for normal enteric ganglion development, whereas their absence or dysfunction may also have a role in the etiology of HD[25,26,27]. The exact mechanisms underlying neural crest cell migration failure and premature death of ganglion cells in HD are still unknown, but complex genetic component play a role.

Gene mutations implicated in the pathogenesis include several proteins that modulate this immigration, differentiation and survival of the neural crest cells. Butler Tjaden and colleagues reported that mutations in the genes - RET, GDNF, GFRα1, NRTN, EDNRB, ET3,

ZFHX1B, PHOX2b, SOX10, and SHH are present in approximately 50% of HD patients [28]. Some patients with HD show mutations in more than one gene.

RET Proto-oncogene & RET/GDNF/GFRα1 Signaling System:

It is a major gene located on chromosome 10 implicated in the pathogenesis of HD with heterozygous mutations, found in 50% of the familial cases and 15–20% of isolated cases [28, 29]. Penetrance of the RET mutations is incomplete and sex dependent. RET mutation is most frequently associated with long segment HD and Total colonic aganglionosis. *RET/GDNF/GFRα1* signaling pathway is one of the primary pathways, which promotes the survival of neurons, mitosis of neuronal progenitor cells, and differentiation of neurons and neurite extension [30]. RET receptor is the signaling component of receptor complexes with four ligands named glial derived neurotropic factor (GDNF), neurturin (NTN), artemin (ATM), and persephin (PSP)[23,30] (Fig 1: A&B). The complete receptor complex includes the RET receptor tyrosine kinase and a glycosylphosphatidylinositol- anchored binding component (GFRα1-4) [23, 31]. Iwashita et al. noted that the glial cell line-derived neurotropic factor receptor

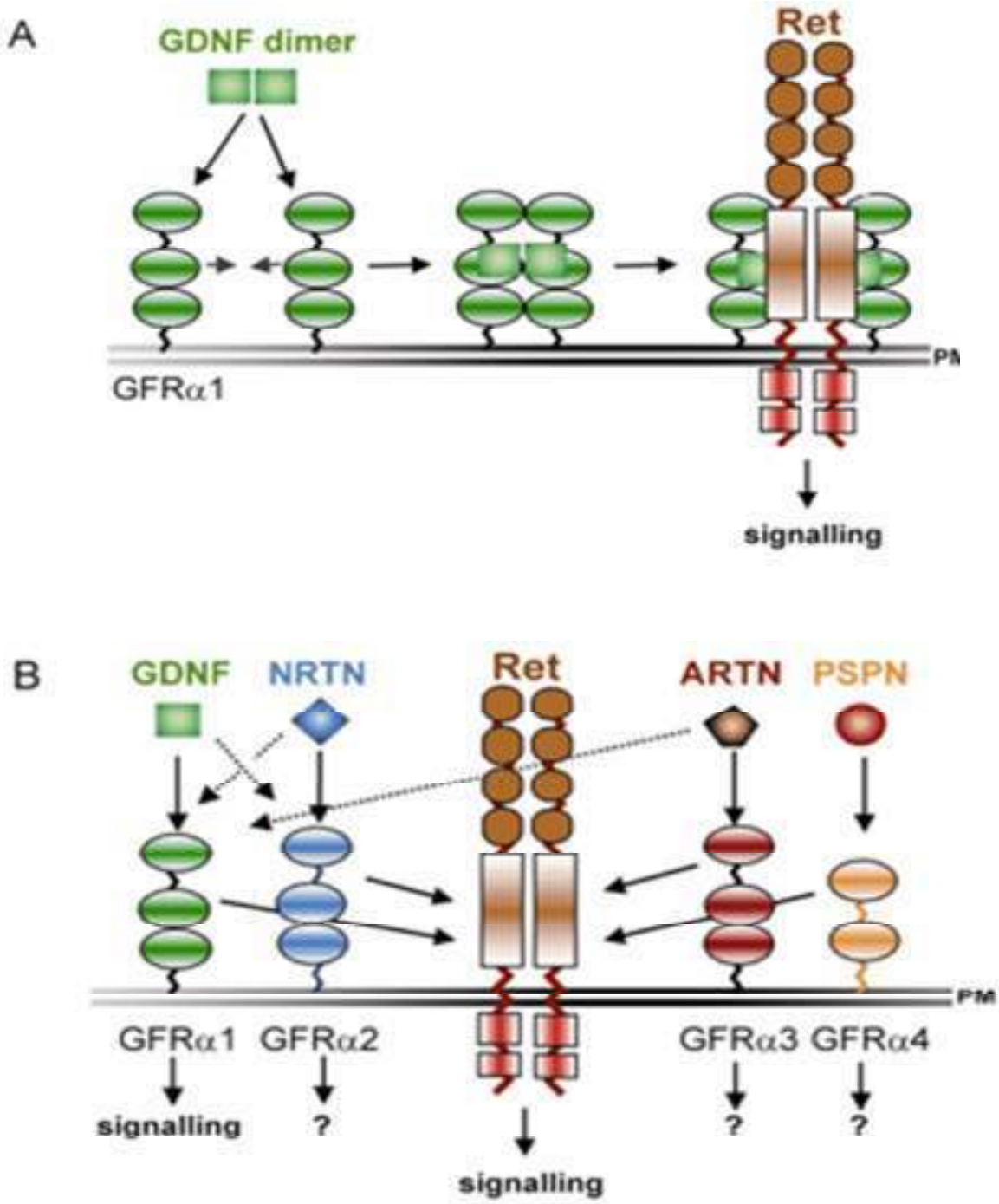


Figure.1: GDNF family ligand (GFL) interaction with their receptors

(A) a dimer of GDNF brings together two molecules of GRFα1. This complex of RET leading to transphosphorylation of their tyrosine kinase domains. (B) All GFLs activate RET tyrosine kinase via different GFR alpha receptors [33].

(GDNF) RET is necessary for neural crest stem cell migration in the gut [32]. Any disturbance of GDNF/GFRα1-mediated signaling leads to the failure of ENS development. Mutations involving the RET gene, GDNF and its co receptor GFRα1 all are responsible for HD pathogenesis.

ENDOTHELIN Signaling Pathway:

The endothelins (EDN1, 2 and 3) are intercellular local messengers that act through the cell surface receptors, EDNRA and EDNRB [30]. EDN is initially expressed in an immature form. It is processed to an active peptide by the enzyme, endothelin converting enzyme 1 (ECE1) [30, 34]. EDN3 and EDNRB have a role in the migration and development of the ENS. Several studies suggest that the downregulation of EDN3 expression may play a role in the pathogenesis of Hirschsprung's disease in the sporadic cases [35, 36].

SOX10 gene:

The SOX10 (sex determining region Y-box) gene is a member of the SRY-related family of transcription factors. It is expressed in neuronal crest derivates that contribute to the formation of the peripheral nervous system during embryogenesis [37]. Mutation in SOX10 is responsible for distal aganglionosis. There were two phenotypes identified PCWH [38] (severe form) and Waardenburg-Shah syndrome

type 4 [39, 40] (WS4- milder form) are caused by two distinct molecular mechanisms.

PHOX2b gene:

Paired - like homeobox 2b gene encodes a transcription factor that regulates *ret* expression and thus it is essential for ENS development [41]. Heterozygous mutations cause a complex dysautonomia, associating Hirschsprung's disease, Congenital Central Hypoventilation Syndrome (CCHS) and Tumors of the Sympathetic Nervous System (TSNS) in various combinations [42].

ZFHX1B gene:

ZFHX1B (zinc finger homeo domain transcription factor) is also known as SMAD interacting protein 1 (SMADIP1/SIP1). The observation of a translocation involving the ZFHX1B locus (2q22) in a patient with Hirschsprung's disease is associated with (Mowat-Wilson syndrome) syndromic form of Hirschsprung's disease [43].

Mutations in the other minor genes like KIAA1279, SHH and etc also involved in HD pathogenesis.

Apart from genetics, Interstitial cells of Cajal, Smooth muscle, Pacemaker cells connecting enteric nerves and Extracellular Matrix also

play a role in the pathophysiology. It suggests that HD's pathophysiology is not limited to cells normally present within the enteric ganglia, alone.

ROLE OF ENZYMES IN PATHOGENESIS:

Nitric Oxide Synthase (NOS) & NADPH - Diaphorase:

Nitric oxide is the major inhibitory non-adrenergic non-cholinergic neurotransmitter in the gastrointestinal tract that mediates smooth muscle relaxation [44]. Nitric oxide is synthesized by the activation of neuronal NOS. Increasing number of this nitrergic nerve fibres are seen in the circular muscle [45]. NOS is abundant in normal colon and ganglionic bowel of HD. In contrast, NOS is selectively absent from the plexus area and from the musculature of aganglionic bowel in HD. Many studies indicate that there is impaired NO synthesis in the aganglionic bowel in HD and this deficiency could prevent smooth muscle relaxation, thereby causing the lack of peristalsis in HD [46, 47]. Nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH- D) is seen in the same location and has been shown to have identical function as that of NOS [48, 49].

Acetyl Choline Esterase (AChE):

AChE gene give rise to various forms of acetylcholine esterase. Hydrophobic G4 form (AChE-G4) is seen in differentiating embryonal

cells, neural crest cells and in the synapses of the central nervous system. In HD there is increase in G4 levels of AChE, which was first reported in rectal suction biopsies by Boston et al [50]. The reason behind why acetylcholine esterase levels are increased in HD is complex. Physical stress, psychological stress, or chemical stress, denervation and electric activity all can increase AChE. It's increase correlate with hypertrophic nerves. These hypertrophic parasympathetic nerves are coarse fibres that develop in muscularis propria and also in the lamina propria. AChE activity is the usual marker of cholinergic nerves and has become a widely accepted technique for diagnosis of HD.

Interstitial cells of Cajal (ICC):

Santiago Ramon y Cajal, a Spanish Nobel Laureate physician and neuropathologist, in the year 1893, first described this as the cell located between smooth muscle and nerve endings. Based on this location he named it as "interstitial"Cells of Cajal. ICCs are modified smooth muscle cells and pacemakercells of the GIT. It consists of a thin cytoplasm, a nucleus that is large and oval and has dendritic like processes. In HD, ICCs, however, are reduced in number and disrupted in pattern, but they are not totally absent from the aganglionic region of the colon [51].

Mast cells (MC) are responsible for various inflammatory processes and produce nerve growth factors, there by influencing on the clinical course of Hirschsprung's disease.

SYNDROMES ASSOCIATIED WITH HD:

I. *NEUROCRISTOPATHIES* -

WAARDENBURG SYNDROME TYPE 4:

Waardenburg syndrome (WS) is a human genetic condition characterized by defective melanocyte function (with pigmentation anomalies of the skin, hair and iris), cochlear sensorineural deafness and craniofacial abnormalities. It occurs in association with intestinal aganglionosis as the uncommon Shah-Waardenburg subtype (WS4). Genes implicated are SOX10, EDN3 and EDNRB.

CONGENITAL CENTRAL HYPOVENTILATION SYNDROME (CCHS):

CCHS or Ondine's curse is an uncommon syndrome occasionally associated with HD (14–20% of cases), as well as with tumors of neural origin and autonomic dysfunction HD-CCHS (Haddad's syndrome). They have a heterozygous mutation in RET, EDN3, GDNF, or BDNF. It is mostly associated with long-segment aganglionosis. Other features

include impaired ventilatory response to hypercarbia and hypoxemia, tonic pupil, neuroblastoma, ganglioneuromas and ganglioneuroblastomas.

MULTIPLE ENDOCRINE NEOPLASIA TYPE 2 (MEN 2):

It is an autosomal dominant disorder characterized by parathyroid hyperplasia, medullary thyroid carcinoma and pheochromocytoma. This disease is a result of RET gene mutations and it is associated with Hirschsprung's disease. It increases the cancer disposition in HD individuals.

II. *NON NEUROCRISTOPATHIES –*

GOLDBERG-SHPRINTZEN SYNDROME:

It is an autosomal recessive condition. Hirschsprung's disease is seen in this syndrome along with cleft palate, colocoma, microcephaly, facial dysmorphism and mental retardation.

BARDET-BIEDL SYNDROME:

It is a autosomal recessive condition. Features of this syndrome include mental retardation, renal abnormalities, progressive pigmentary retinopathy, obesity, hypogenitalism, postaxial polydactyly and it is associated with Hirschsprung's disease in about 2% of the individuals.

BRESEK/BRESHECK SYNDROME:

It is a rare X-linked multiple congenital malformation characterized by Brain anomalies, Retardation, Ectodermal dysplasia, Skeletal deformities, Hirschsprung's disease, Ear/Eye anomalies, Cleft palate/Cryptorchidism and Kidney dysplasia.

KAUFMAN-McKUSICK SYNDROME:

In this syndrome HD associated with Hydrometrocolpos, postaxial polydactyly, congenital heart defect.

Aside from, number of unusual hereditary syndromes has been reported in patients with HD, which includes Smith-Lemli-Opitz syndrome, Cartilage-hair hypoplasia syndrome Mowat-Wilson syndrome, Down syndrome and syndromes with HD and distal limbs anomalies [52].

CLINICAL PRESENTATIONS:

Hirschsprung's disease is the disease of the new-born. It is suspected if the child doesn't pass meconium within 48 hours after birth. This classical presentation may be absent in 6% to 42% of the patients according to Teitelbaum et al. Other symptoms are signs of lower intestinal obstruction such as abdominal distension and bilious vomiting. Hirschsprung's disease can also first present itself as enterocolitis and

sepsis, mainly in the more extended forms. The neonate with HD is usually a full-term baby. The diagnosis of HD in the newborn period is made in 90.5% of patients. After the new-born period, the most common presentation is constipation, abdominal distension accompanied with failure to thrive. But all these are not specific.

The clinical differentials at this stage include,

- *Obstructive lesions* - Colonic atresia or duplication, meconium plug syndrome, anorectal anomalies and malrotation etc..
- *Neurological Lesions* - Intestinal neuronal dysplasia (IND), hypoganglionosis and similar conditions.
- *Endocrine and metabolic disorders* causing obstructive symptoms.

Hypoganglionosis:

Isolated hypoganglionosis represents only 5% of intestinal neuronal malformations [53]. Only full thickness biopsies are reliable for establishing the diagnosis of hypoganglionosis, as analysis of the intramural plexus is essential. In this, number of ganglion cells per nerve unit in the myenteric plexus is decreased and the interganglionic distance is significantly increased [54, 55]. Usually less than 14 ganglion cell per centimetre and decreased nuclear diameter of ganglion cells [56]. In

addition, it is also characterized by a reduction in the number of LDH-positive nerve cells in the myenteric plexus and a scarcely developed network of parasympathetic nerve fibres in the circular and longitudinal muscles with a low AChE activity.

Intestinal Neuronal dysplasia:

Intestinal neuronal dysplasia (IND) is a malformation of the ENS and is a controversial entity. In most patients, IND is clinically indistinguishable from Hirschsprung's disease at presentation. Some investigators have reported that 25–35% of patients with HD have associated IND [57]. Two forms are recognised: IND A and IND B.

IND type A (Hypoganglionosis) is characterized by the lack or immaturity of sympathetic innervation of the myenteric plexus. This condition is extremely rare.

IND type B (Hyperganglionosis) is a developmental abnormality of the submucosal plexus characterized by the presence of *"giant ganglia"* in the submucosa, *ectopic ganglion cells* in the lamina propria, muscularis mucosa and inner circular muscle. Abnormal increase in acetylcholinesterase positive nerve fibres with in the lamina propria and around submucosal blood vessels are also present [58]. Normally innervated colonic mucosa contains 4 ± 2 ganglion cells where as IND

type B show 10 ± 2 ganglion cells per ganglia [59]. If 15–20% of the submucosal ganglia in the rectal biopsy are giant ganglia, a diagnosis if IND type B is appropriate. The nerve cells in the giant ganglia are significantly smaller and anisomorphous compared with those in normal biopsies. However, the reproducibility of the histological diagnosis remains doubtful.

The histopathological findings in HD are explained in detail below.

CLASSIFICATION OF HD:

1. *Short segment disease (Classic HD)* – the aganglionic segment does not extend beyond the upper sigmoid colon. Most common form of HD constitute 75% to 80% involves Distal sigmoid colon and rectum.

2. *Long segment disease* - aganglionosis extends to the splenic flexure or transverse colon .The disease extends beyond the splenic flexure. It comprises about 15% to 20%.

3. *Total colonic aganglionosis* (*TCA*) – aganglionic segment extends from the anal canal to at least the ileocecal valve but does not extend more than 50 cm beyond the ileocecal valve. The incidence of this form is 5% to 10%. Male to female ratio is equal in TCA.

4. *Ultra short segment disease* - UHD is defined as an aganglionosis in the anal canal, proximal to the pectinate line about 3–4 cm in length. An absolutely accurate diagnosis of UHD is only possible with an enzyme histochemical AChE reaction [60].

5. *Total intestinal aganglionosis* - Total intestinal aganglionosis with absence of ganglion cells from duodenum to the rectum is the rare form of HD [61].

6. *Zonal aganglionosis* – zonal or segmental (skip) aganglionosis in which the absence of ganglion cells is patchy, so the surgical correction may fail [62].

Depending on the time in which the neural crest cell arrest takes place the involved segment varies. If it is inhibited by 8th week of gestation then the affected region is the entire colon. Affected segment is descending colon along with recto sigmoid if there is arrest by 9th week of intra uterine life. Only the recto-sigmoid is affected if there is delay in the arrest as late as 10-12th week of intrauterine life. Long segment aganglionosis is the result of early arrest. The aganglionosis is continuous and uninterrupted until the proximal transitional zone is reached.

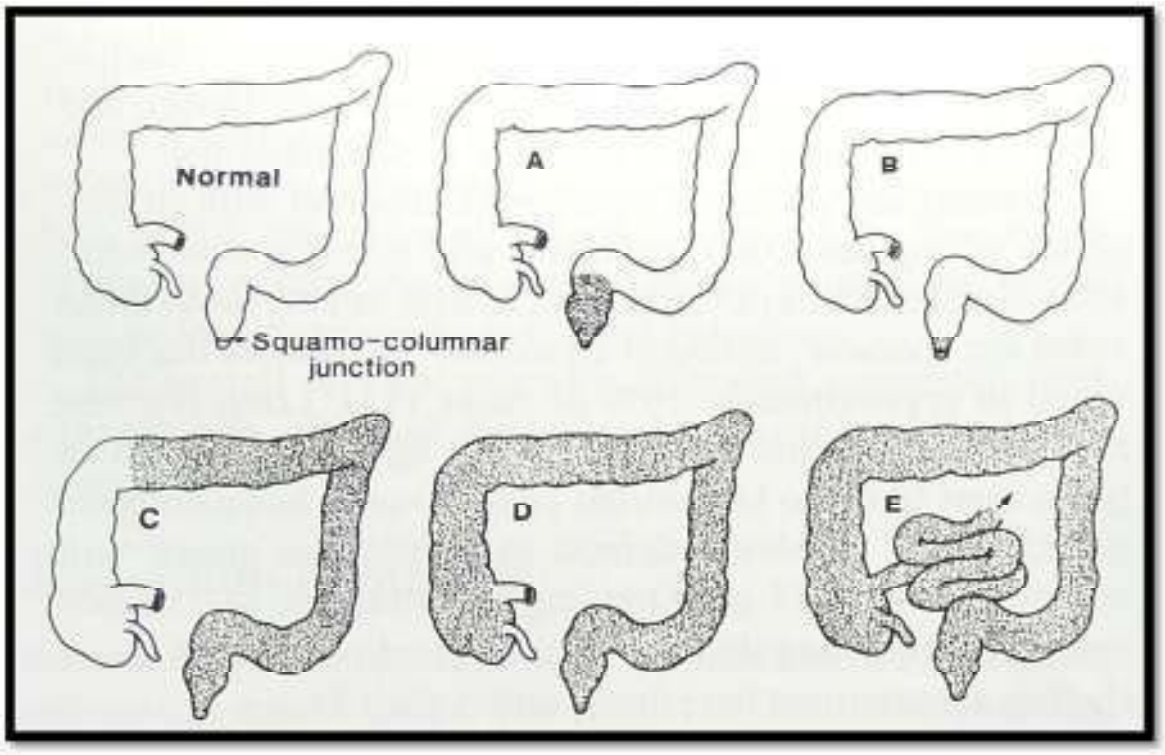


Figure 2: **Extent of HD** (stippled area), **A**: Short segment HD, **B**: Ultrashort segment HD, **C**: Long segment HD, **D**: Total colonic aganglionosis, **E**: aganlionosis extends into distal small bowel. (Adapted from Stocker& Dehner's pediatric pathology, 3rd edition, chapter 14, page no 594).

INVESTIGATIONS:

Ano rectal manometry:

Rectal inhibitory reflex is elicited by distending the rectum and monitoring the response of the internal anal sphincter. This test is reliable only after 12th perinatal day. Two to sixty ml of air is insufflated to elicit this response. Internal sphincter pressure of 5mm Hg for 2-5 seconds is

considered to be normal after distention. This recto anal inhibitory reflex is absent in HD [63].

It has limitation like false negative value in case of probe displacement, difficult in case of un-cooperative children and false positive results in case of insufficient inflation and air leak.

Radiological diagnosis:

Plain X- ray finding that clinch the diagnosis include absence of gas shadow in the narrowed rectum and dilated proximal colon. Supine and lateral decubitus plain films are performed routinely. The level of obstruction may be indicated by the presence of undistended colon or rectum. *Barium enema* confirms diagnosis with cone-shaped or funnel-like appearance of the transitional zone between the distended proximal bowel and the narrowed aganglionic distal segment.

It has disadvantages like radiation exposure and when rectal wash is given, the dilated portion is decompressed masking the typical picture.

ROLE OF HISTOPATHOLOGY IN DIAGNOSIS:

Although clinical and radiological correlations are essential, the "gold standard" diagnostic modality is histopathological examination.

Specimen:

Rectal biopsy whether it may be suction biopsy or full thickness biopsy is the most common procedure performed to diagnose HD. Sometimes intra operative rectal biopsy is carried out for the analysis of enzyme histochemistry using frozen sections.

Rectal suction (mucosal) biopsy (RSB) remains the favoured approach in neonates and young infants, as contemporary biopsy guns yield diagnostic specimens (3.5–5 mm in diameter, with at least 2 mm of submucosa) with minimal morbidity. The suction device relies on vacuum to elevate and excise an adequate tissue. RSB can be done at bedside without the need for general anaesthesia or suturing and is less traumatic to the patients. Diagnosis of aganglionosis is made in either plexus (submucosal or myenteric), since at any level aganglionosis of both plexuses is synchronous. Simplicity and absence of complications strongly favour RBS as the procedure of choice for the diagnosing HD nowadays [64].

Full thickness biopsy is preferred for older infants and children [65], as RBS is more prone to produce inadequate specimens, probably due to over distention of the rectal wall, mucosal edema, or increased fibrous tissue, making it difficult for the suction device. It may be open or endoscopic guided biopsy which aims to obtain a larger volume of tissue

from a well-controlled location. It requires general anaesthesia and may be followed by complications like bleeding, perforation, and scarring.

Also diagnosis of aganglionosis is made in either plexus, since at any level aganglionosis of both plexuses is synchronous. So, many institutions prefer rectal suction biopsy rather than full thickness/ seromuscular biopsy for primary diagnosis of HD.

Intra operative biopsy for frozen section with AChE enzyme study can helps in immediate diagnosis and guides the surgeon on the level of transection of the bowel in the same sitting.

Apart from initial diagnosis, biopsies may be performed several times like, at enterostomy, again during pull through surgery, and sometimes again due to persistent constipation.

Diagnostic accuracy depends on the **adequacy** of the specimen and **biopsy site**. Biopsy should be taken 2-3 cm proximal to the pectinate line (transition between rectal and squamous mucosa).A number of studies have demonstrated that the anal canal and the distal 1-2 cm of rectum is normally hypoganglionic or aganglionic, and a justifiable concern exists that sampling of these areas may lead to a false positive impression of aganglionosis[66, 67]. Also, biopsies taken too high may miss very short segment HD limited to the distal rectum.

According to Aldridge and Campbell study, at least two biopsies should be obtained [66]. However, no universal standard exists, and some practitioners recommend more extensive sampling. A minimum, a diagnostic biopsy should measure approximately **3mm in diameter** and have at least one-third to half of submucosa. An accepted rule of thumb is that in a well oriented biopsy the portion of submucosa sampled should be at least as thick as the overlying mucosa.

Unsatisfactory or inadequate biopsy:

Biopsies are reported inadequate when,

1. No or very minimal sub-mucosa is present; 2.Sub-mucosa is occupied by a lymphoid follicle; 3.Specimen is decomposed; 4.Specimen reveals stratified squamous epithelium (‘low biopsy’ from anal canal).

When a **Resected bowel specimen** is received after surgery it is processed for H&E examination. The slides are evaluated for presence of ganglion cells at the proximal resected margin. The presence of ganglion cells in proximal margin ensures the adequacy of resection while its absence helps in guiding the surgeon to select the colostomy site.

Microscopic features:

The characterized finding of HD is aganglionosis in both the submucosal and myenteric plexus and presence of hypertrophied nerves in the submucosa. So, deep insight about normal bowel morphology and ganglion cell, nerve plexus distribution in various layers of intestine is necessary.

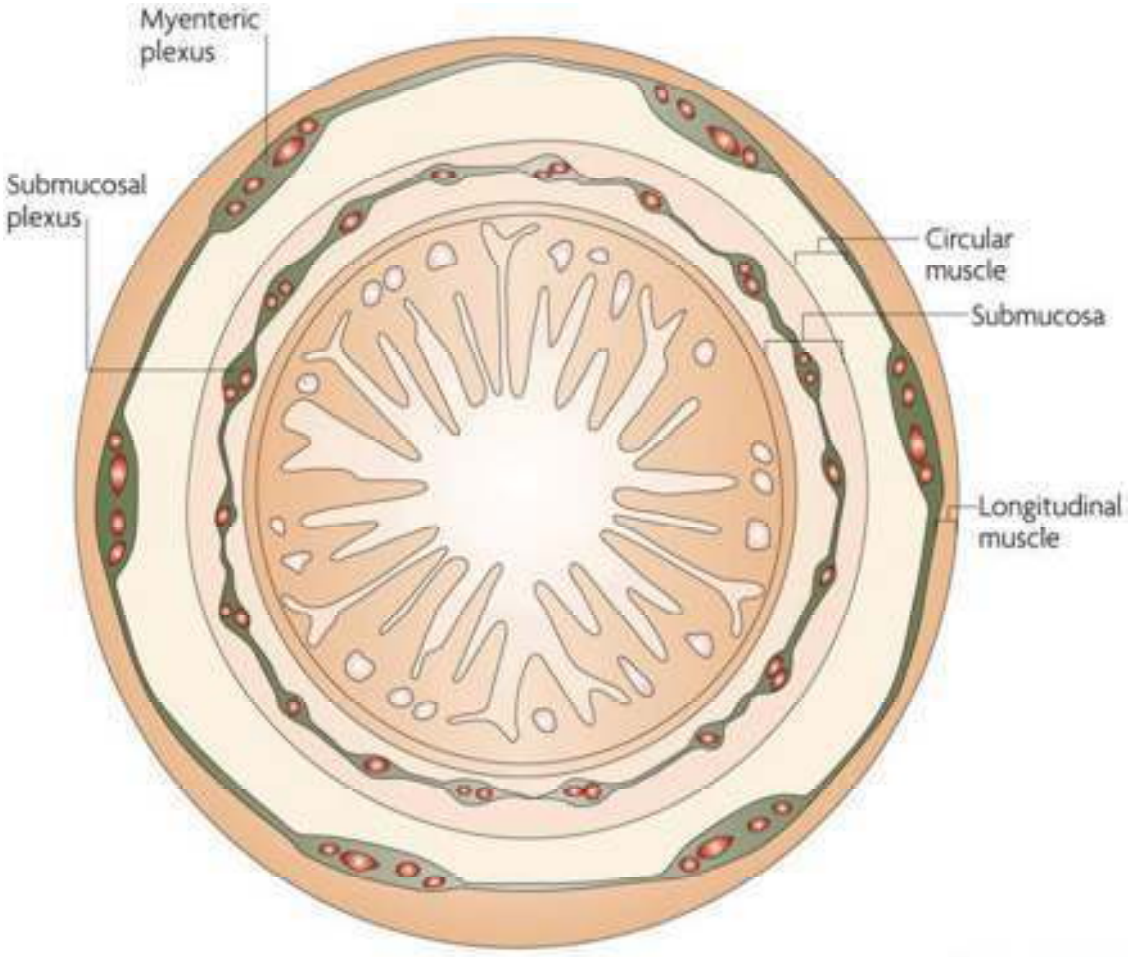


Figure 3: Diagrammatic representation of cross-section of bowel layers and nerve plexus distribution.

The intestine is a tubular structure and has four distinct functional layers (from lumen to outside) – Mucosa, Submucosa, Muscularis propria and Serosa. The mucosa is made up of lining epithelium, lamina propria and muscularis mucosa. Muscularis propria has inner circular and outer longitudinal muscle layers (Fig 3).

There are three plexuses in the intrinsic nervous system which includes the inner **Meissner's** (Submucosal) **plexus** found only in the small and large intestine which lies just beneath the muscularis mucosa. The deep submucosal plexus or **Henle's plexus** lies close to the surface of the circular muscle layer and the outer **Auerbach's** (Myenteric) **plexus** (Figure: 6) running the full length of the gut between the longitudinal and circular smooth muscle layers consists of unmyelinated nerve fibres, clusters of rounded Schwann cells and large ganglion cells around the perimeter of nerve fibres.

GANGLION CELLS:

Ganglion cells are contained within "neural units" first described by Yunis[68]. They are typically large polygonal cells having amphophilic to basophilic cytoplasm which contains Nissl's granules with eccentric nuclei and prominent nucleoli. Normally, one to five ganglion cells are seen in clusters at equi-distance for every 1mm of normal rectal mucosa [66] (Mean myenteric ganglion cell density in Colon is 7ganglion

cell/mm). Their arrangement in clusters and their association with nerves facilitates recognition. In neonates and premature infants, the ganglion cells are often smaller, without a recognisable nucleoli and cytoplasmic Nissl's granules are also absent, making its identification more difficult particularly in the submucosal plexus.

Additional H&E findings in HD:

Thickened nerve fibres:

Absence of ganglion cells in an adequate biopsy is diagnostic of HD and the presence of numerous unusually large, prominent submucosal and myenteric Schwannian nerve elements are regarded as useful supportive feature in making diagnosis. These hypertrophic nerve fibres more than **40µm** in diameter strongly correlate with abnormal innervation/aganglionosis [69]. But these hypertrophic nerve fibres are less common in ultrashort segment HD, long segment HD (LHD), TCA and also in young infants [70, 71].

Histological grading system for HD:

Teitelbaum et al. proposed in 1989 that the presence of HD implies an alteration in the mucins of the large bowel with associated mucin retention and crypt dilatation [72]. They proposed a histological grading system ranging from normal to gross abnormality using both histological

features and the feature of mucin retention which is unique to HD and also cystic fibrosis (Table:1). Using this grading system we can identify patients with histopathological **Hirschsprung's disease associated enterocolitis** (HAEC) in the suction rectal biopsies before they became symptomatic. HAEC is a serious complication that can occur pre- or post resection of the aganglionic bowel and clinically manifest as abdominal distension and diarrhea, usually accompanied by fever and often bloody stools. Grade III or higher in this grading system predict the development of enterocolitis in patients with HD [72].

Table 01: Histological grading system for Hirschsprung's disease

Grade	Histopathological features
0	No abnormalities
I	Crypt dilatation and mucin retention
II	Cryptitis or two crypt abscesses
III	Multiple crypt abscesses
IV	Fibrinopurulent debris and mucosal ulceration
V	Transluminal necrosis or perforation

Before issuing a definitive report, it is necessary to examine serial sections of paraffin embedded tissue to establish the diagnosis of aganglionosis. In practice, it is recommended that at least six slides, each bearing a ribbon of 6 to 10 serial sections should be evaluated when the ganglion cells are absent.

Limitations of H&E in diagnosis of HD:

Rectal suction biopsy (RBS) is becoming the procedure of choice for obtaining specimens for the initial diagnosis of HD because of its low morbidity. Morphologic immaturity of ganglion cells especially in neonates and infants, and sparse distribution of ganglion cells in the submucosal plexus need careful evaluation of many sections (more than 50) before a biopsy can be interpreted as negative for ganglion cells. It is a time consuming process when routine H&E is used alone [73]. Also, immature morphology of ganglion cells in neonates can be confused with endothelial cells or neuronal cells leads to false negative diagnosis. Sampling error is another major factor in wrong diagnosis. False positivity can occur if the site of biopsy is too distal due to physiological paucity of ganglion cells. Superficial biopsy without enough submucosa can also result in patient being wrongly diagnosed as HD.

In order to overcome these limitations, many ancillary techniques have been proposed to improve the detection rates, reduce false positivity and increase the reproducibility and the ease of diagnosis.

ANCILLARY TECHNIQUES:

Nowadays, full thickness specimens are being replaced by suction or mucosal rectal biopsies in the diagnosis of HD. Interpretation is more difficult in these specimens using H&E alone. Ancillary techniques like enzyme histochemistry and IHC staining may encourage more and more pathologists and surgeons to prefer suction biopsies instead of the more invasive full thickness specimens.

I. HISTOCHEMICAL STAINING

Acetylcholinesterase (AChE) enzyme study:

AChE enzyme study is the most widely accepted histochemical stain to diagnosis HD. The principle of the histochemical staining is based on the original technique of Karnowsky and Roots [74]. It requires examination of fresh, unfixed specimens. The specimens are subjected to 10-12 μm thick sections for proper highlight of nerve fibres. Normally innervated intestinal mucosa does not generally stain for AChE [75]. So, the presence of increased density and thickness (coarse) of AChE positive cholinergic nerve fibres in the lamina propria between the crypts and

muscularis mucosae is regarded as highly specific for the HD diagnosis [76]. Demonstration of the AChE-positive nerves in the aganglionic zone appears to correspond with the severity of the clinical presentation and degree of obstruction in HSCR as well according to Garrett et al [77].

While all techniques look at the absence of ganglion cells, this is the only ancillary technique where a positive result is diagnostic of HD. The main advantage of this technique is its quick diagnosis, rapid procedures have been developed to reduce the time to 5-10 minutes [78, 79], instead of traditional protocol for AChE staining which requires approximately 90 minutes. Hence definitive treatment can be planned in the same setting.

However, the technique is not without limitations and they have been addressed in many studies [80, 81].

- Needs fresh tissue sample, cryostat, interrupting the usual workflow of a surgical pathology laboratory.
- Possible need for extra biopsy for H&E
- AChE is a unique histochemical stain used only for HD and has cost implications in using it.

- It requires freshly prepared reagent [82] and is a difficult histochemical stain to perform.

- Results are influenced by improper tissue handling and staining quality.

- False negative results can occur especially in young infants [83] or ultra short segment HD and TCA [84, 85]. In TCA, AChE staining pattern may appear almost normal due absence of submucosal hypertrophic nerves, limiting its application.

- AChE studies are subjective as they require both qualitative and quantitative assessments [86] and studies have also shown more disagreements in the interpretation of AChE stain [21].

Other histochemical stains:

A variety of histochemical stains like Lactate dehydrogenase (LDH), Alpha-naphthyl esterase, NADPH-diaphorase which highlights the enzyme present in ganglion cells and Toluidine blue and others can support the diagnosis of HD.

LDH is a complementary enzyme study for HD. However it plays a major role in diagnosis of Hypoganglionsis and Intestinal neuronal dysplasia.

Due to the absence of ganglion cells in the aganglionic segment of HD patients, there is an absence or marked reduction of **NADPH diaphorase** positive nerve fibres in both muscle layers and the muscularis mucosae. The typical hypertrophied nerve trunks appear weakly stained [87].

Toluidine blue is a metachromatic dye, has an affinity for nucleic acids, and therefore binds to nuclear material with a high DNA and RNA content, and to chromatin or Nissl substance in the cytoplam. It selectively stains ganglion cell nucleus blue and cytoplasm light blue allowing for easy identification of these cells and can be a very useful adjunct to H&E [88].

II. IMMUNOHISTOCHEMICAL TECHNIQUES:

As a means to improve the diagnostic accuracy of HD over H&E and to overcome the limitations of AChE staining, other reliable diagnostic methods like IHC has been tried to facilitate diagnosis. In recent years, the use of several markers have been attempted to aid the diagnosis of this disorder, including S-100 protein [89, 90], neuron specific enolase (NSE) [90], glial fibrillary acid protein (GFAP) [91, 92], glucose transporter 1 (GLUT-1) [93], microtubule associated protein 5 (MAP-5) [12], CD56, Synaptophysin, Chromogranin A, Cathepsin D, Vimentin, Bcl2, and others [94]. IHC staining has the advantage of using sections

from paraffin embedded biopsies, thus eliminating the need to obtain extra biopsies. Also this technique is available in most pathology laboratories.

Synaptophysin and Chromogranin A show lower sensitivity, specificity and reactivity to ganglion cells, making them less reliable and less useful diagnostic markers for Hirschsprung's disease and allied disorders, although synaptophysin shows strong staining of myenteric plexuses which makes it a good diagnostic marker for hypertrophic or hypotrophic changes of extrinsic nerve fibres [95].

S100 immunostain is a nerve sheath marker used to highlight the nerve hypertrophy. Besides S100 can be utilized as an indirect marker of the presence ganglion cells which identifies the ganglion cells as prominent negatively stained cells and surrounded positive Schwann cells and nerve fibres. They can highlight the presence of hypertrophic nerve fibres and presence of ganglion cells bodies by their lack of staining.

NSE IHC stains the ganglia intensely, helping in the recognition of small immature ganglion cells.

Several other immunohistochemical markers have been tested, but most of them have limitations for use in daily practice [80].

CALRETININ:

Calretinin is a 29 kDa calbindin and a member of EF hand protein. It is a Vitamin D dependent calcium binding protein, palys a role in various cellular functions including message targeting, physiological buffering of excess intracellular calcium ions and gives protection against calcium ion overload [96]. Calretinin is abundantly expressed in neurons including retina (hence the name) [97], cortical inter neurons. Calretinin has been shown to have a broad tissue distribution like mesothelium, Sertoli & Leydig cells of testis and mast cells etc which also includes strong expression in neural elements [98]. Nerve cell bodies (ganglion cells) and nerve fibres in both submucosa and myenteric ganglia in the human gastrointestinal tract are immunopositive for Calretinin [99]. Loss of Calretinin immunoreactivity in ganglion cell and nerves has been documented by many authors, to be characteristic of HD.

McConalgue *et al* in 1994 was the first to show in the large intestine of guinea pigs that Calretinin immunoreactivity highlights different neuronal populations[100]. Since then a number of comparative studies have been published on the expression of calretinin in HD and its utility as a marker for assisting in the diagnosis of HD. Barshack et al utilized this Calretinin reactivity to diagnose HD in the year of 2004. Calretinin immunorectivity was studied in ten large bowel full thickness

specimens (a total 54 paraffin wax blocks) from patients with classic rectosigmoid HD. Calretinin was not expressed in aganglionic segments of HD and associated nerve fibres, whereas both ganglion cells and nerve fibres were immunopositive in ganglionic HD segments and in normal colon. The transitional zone showed a broad spectrum of histomorphological and immunohistochemical patterns of calretinin expression [101].

Kapur et al compared calretinin versus acetylcholinesterase (AChE) in normal and HD patients concluding that calretinin is superior to AChE as an adjunct diagnostic method for HD. Multiple observers independently reviewed calretinin IHC and AChE sections of suction biopsies from 14 HD and 17 controls. There were 2 misdiagnoses and more disagreements in the interpretation of AChE-stained sections, but calretinin IHC showed no misdiagnoses or discrepancies [81]. They concluded that calretinin IHC to be a better alternative to AChE as an adjunct in diagnostic method for evaluating suction rectal biopsies for HD [81]. One major problem reported in cases of AChE interpretation was high interobserver disagreements. Calretinin IHC substantially reduces this problem and appears to be a superior alternative to AChE. Also they observed punctate axonal immune staining of hypertrophied nerve fibres in few HD cases in their study.

Similar observation was published in 2009 by Guinard-Samuel *et al*. They evaluated the calretinin immunostaining as a primary diagnostic tool on a large series of suction rectal biopsies [102] .They retrieved 131 biopsies carried out for suspicion of HD in children and infants to compare the accuracy of calretinin immunohistochemistry with the standard method (histology and acetylcholinesterase staining). In their study, calretinin immunohistochemistry enabled the diagnosis of all HD diagnosed by the standard technique, except for one patient who had a weak positive immunostaining in some nerve fibers (false negative case). It is important to note that 12 additional cases initially considered as suspicious for HD using the standard technique were accurately diagnosed by calretinin immunohistochemistry. The authors also noted faint positive staining of some nerve fibers with Calretinin IHC but with no other staining (ie, muscularis mucosae, lamina propria or nuclear staining) in authentic HD. To avoid this potential and exceptional pitfall, authors suggested, Calretinin should be used in combination with standard histology and that it might be more accurate than acetylcholinesterase in detecting aganglionosis [102].

Hiradfar M et al in 2012, in their study evaluated 80 paraffin blocks, previously fixed in 10% buffered formalin. Those comprised 30 blocks from the aganglionic zone, 30 from the ganglionic zone and 20

blocks from control group. Calretinin immunoreactivity and pattern of staining for ganglion cells (nuclear and cytoplasmic) and also nerve fibres in different layers of bowel (lamina propria, muscularis mucosa, submucosa and muscularis propria) were evaluated in IHC stained slides. Calretinin IHC showed a sensitivity and specificity of 93.3% and 100% respectively for diagnosing HD in full thickness biopsies of intestinal wall. The positive predictive value and negative predictive value were 100% and 93.8%. There was no false- positive or false negative results based on calretinin immunostaining in submucosa. They concluded it can be used on suction rectal biopsies as a reliable adjunctive method to diagnose HD [103].

An another study evaluating calretinin staining pattern in both ganglionic and aganglionic segment showed that Calretinin IHC stain highlighted the ganglion cells, submucosal and subserosal nerve trunks in normal rectum. Also, it is expressed in a linear, granular pattern, in the nerve fibrils of the superficial submucosa, muscularis mucosae and lamina propria. However, Calretinin immunohistochemical stain was negative in the aganglionic segments of bowel examined. Nerve fibrils in lamina propria, muscularis mucosae or superficial submucosa stained negative for Calretinin. Occasional cells, possible mast cells, expressed calretinin in the submucosa both in ganglionic and aganglionic segments,

which can be used as an internal control for assessing quality of staining. Unexpectedly, two of 13 HD biopsy specimens which showed no ganglion cells and no Calretinin immunoreactivity in any of the compartments, showed a faint, granular, axonal reactivity in the submucosal hypertrophied nerve trunks [104]. These findings were also observed in the nine pull through specimens in which calretinin was performed on the sections showing the transition from affected-to-unaf-fected bowel. No explanation could be provided for this pattern by the authors. Guinard-Samuel et al. believed that this positive staining of some nerve fibers could indicate the beginning of transitional zone [102].

Kannaiyan L *et al.* [104] in their Indian based study of sixty cases including 34 initial full thickness colon biopsy and 26 resected specimens, found that calretinin correlated with H& E examination in both rectal biopsies and the resected bowel specimens. In the rectal biopsy specimens, calretinin also aided in the diagnosis of 15 patients with ambiguous findings and they concluded that Calretinin was extremely useful in solving the suspicious and indeterminate cases of HD. It can serve as a valuable cost‐effective diagnostic aid in the centers where acetylcholinesterase enzyme histochemistry is not available [105].

Mukhopadhyay B et al selected one hundred and five blocks for calretinin IHC. All representative areas including lamina propria,

submucosa, and muscularis mucosae were examined for detection of calretinin positivity by two pathologists blindly. Satisfactory concordance was obtained between H&E reports and calretinin study. Overall sensitivity, specificity, positive, and negative predictive value were 100%, 92.73%, 92.45%, and 100%, respectively [106].

Rakhshani N et al studied a total ninety four cases and compared concordance between H&E diagnosis and Calretinin IHC diagnosis. Results showed that disagreement between calretinin IHC and H&E staining in identification of ganglion cells occurred in 3 cases, whereas disagreement between calretinin IHC and H&E staining in identification of intrinsic nerve fibres occurred in 4 cases. Comparing the values of specificity and accuracy between calretinin and standard histology (H&E), by the Fisher exact test, calretinin presented significantly (*P* value <0.0001) higher specificity and accuracy values than H&E staining. The measure of agreement by Kappa test showed there were high agreement in IHC and H&E staining of Ganglion Cells and it was significant rather than with intrinsic nerve fibres (*P* value <0.0001) [107].

The immunoreactivity of three IHC markers, viz., Calretinin, synaptophysin and chromogranain were compared and recored as 1+, 2+ or 3+ based in intensity of immunoreactivity. Calretinin showed the highest reactivity (mean score of 2.47) for ganglion cells when

compared to other neuronal markers like synaptophysin (0.58) and chromogranin A (0.63) [95].

Another additional benefit of calretinin immunostaining is the presence of positive control in the sections of the submucosa, i.e. some non neuronal cells such as histiocytes and mast cells showed cytoplasmic and nuclear immunoreactivity that can be considered as internal positive control.

Calretinin immunohistochemistry is done on paraffin sections and its interpretation which is based on negative or positive results is much easier [81, 102].

Limitations of Calretinin:

- Calretinin showed only a moderate reactivity for nerve plexus when compared to other neuronal markers like synaptophysin and chromogranin A. [95]
- Several previous reports also have noted the possibility of false negative results in calretinin IHC due to immunostaining in some non neuronal cells [101,102].

- Unlike AChE staining which can provide diagnosis intraoperatively, Calretinin IHC can be done only in processed tissues. Repeat biopsy may be required in case of inadequate sample.

- Because of the phenotypically heterogeneous nature of HD [81], the utility and efficacy of calretinin immunohistochemistry, especially in ultrashort segment HD or transitional zone assessments, remains unexplored. So, calretinin immunohistochemistry should always be used in addition to H&E sections while evaluating for HD thus adding to the cost.

MATERIALS AND METHODS

STUDY DESIGN

The present study is a retroprospective study. Retrospective study period is August 2016- November 2016 and Prospective study period is December 2016- March 2018. A total sample of 30 cases of suspected Hirschprung's disease was analysed.

Ethical clearance for the study was obtained from the Ethics Committee of Coimbatore Medical College, Coimbatore.

STUDY SETTING

The study is undertaken in the Department of Pathology, Coimbatore medical College and Hospital, Coimbatore.

STUDY PERIOD

August 2016 to March 2018

SELECTION CRITERIA

1. Inclusion criteria

- Age: Day1 to 15 years
- Gender: Both sexes

- All clinically suspected cases of Hirschsprung's disease who underwent rectal biopsy or bowel resection.

2. Exclusion criteria

- Ill fixed specimen or specimen not sent in formalin.
- Biopsy specimen which contains the epithelium of the anal canal or skeletal muscle.

SPECIMEN RECEPTION AND PROCESSING:

The study was done in 30 biopsy or resected bowel specimens of clinically suspected Hirschprung's disease, received from Department of Pediatric Surgery, Coimbatore Medical College and Hospital, Coimbatore.

Processing of fresh specimens:

The specimens were received in 10% formalin for routine histopathological examination with a request form containing the necessary history, investigation details and information about the specimen. The specimens were fixed in formalin for 8-12 hours. In case of rectal biopsy specimen, the whole specimen was embedded. Resected bowel specimens were evaluated grossly and representative samples were taken each from distal spastic segment, transitional zone, and proximal

ganglionic region. Sampling of proximal margin is most important and we did circumferential sampling of proximal end.

Sampled specimens were processed in an automated tissue processor (LEICA TP1020) for 16 hours and then embedded in paraffin wax. Blocks were sectioned using microtome for H&E and Calretinin IHC as explained below.

Collection of specimens for retrospective data:

All cases of suspected HD who underwent rectal biopsy or bowel resection between August 2016 and November 2016 were retrieved from pathology records. The blocks and H&E slides of these patients were collected and processed for further analysis. Blocks were cut 4µm thick using microtome for Calretinin IHC staining and for H&E if required.

Hematoxylin & Eosin staining was done in our laboratory using Erhlich's hematoxylin for 10-15 minutes.

Calretinin IHC staining process:

Sections cut from microtome were collected on 'coated' slides, prepared using 2gms Gelatin, 1gm chromalum, pinch of thymol crystal added in 200ml of distilled water and heated at 300˚C for 1min.

IHC staining was performed on paraffin embedded tissues, using the following

BUFFERS AND CONSTITUENTS

- TRIS EDTA BUFFER: p^H – 9

  TRIS buffer salt – 6.05 gms

  Disodium EDTA – 0.74 gms

  Distilled water – 1000 ml

- TRIS BUFFER SALINE: p^H – 7.6 to 8

  TRIS buffer salt – 0.605 gms

  Sodium chloride – 8 gms

  Distilled water – 1000 ml

  1N Hydrochloric acid – 3.5 to 4ml

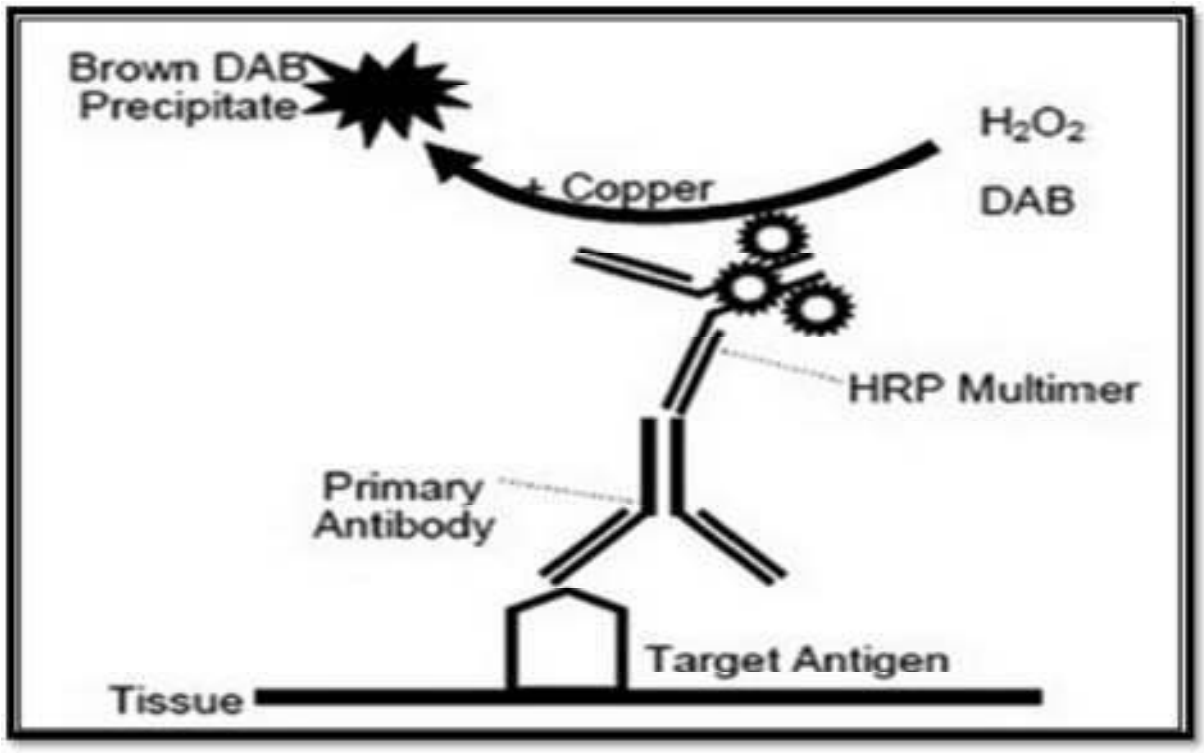


Figure 4: Schematic representation of IHC procedure (Indirect method)

IMMUNOHISTOCHEMISTRY PROCEDURE

1. 4µm sections were taken on the coated slides.
2. Slides were incubated overnight in incubator at 60˚C
3. Tissue sections deparaffinised in Xylene for ten minutes – 3 changes
3. Washed using absolute alcohol for five minutes – 2 changes
4. Washed with tap water for five minutes
5. Rinsed in distilled water for two minutes

6. Antigen retrieval – done by placing the slides in a microwave with Tris EDTA buffer for 20 minutes (ten minutes each at 450˚C and 800˚C).

7. Cooled it in room temperature and then rinsed in distilled water

8. Peroxide block applied on sections for 10 minutes

9. Washing was done in TBS buffer for five minutes – two changes

10. Drained the slide and added Primary Antibody- Calretinin (Rabbit monoclonal Calretinin antibody, Clone EP1798, Bio SB)which was followed by incubation at room temperature in a moisture chamber for one hour

11. TBS buffer wash for five minutes – two changes

12. Slides covered with Secondary antibody – Bio SB - Mouse / Rabbit PolyDetector/ HRP label for 30 minutes.

13. Washed in TBS buffer for five minutes – 2 changes

14. DAB chromogen was diluted with Bio SB buffer substrate and applied for five to eight minutes.

15. Rinsed in distilled water

16. Counterstaining was done with Harris hematoxylin for 1 minute

17. Blueing for five to ten minutes

18. Slides air dried, cleared in Xylene and mounted in DPX.

All biopsies received with clinical diagnosis of suspected HD were included. 22 full thickness biopsies and 8 resected specimens were received during the study period. One ganglionic segment and one aganglionic segment of the 22 biopsies (total 44 paraffin blocks) were processed for each case. In the 8 resected specimens, representative tissues taken from dilated ganglionic, spastic aganglionic and transition zone (total 24 paraffin blocks) were processed. On the whole sixty eight paraffin blocks were studied.

Hematoxylin & Eosin interpretation:

Slides from all 68 paraffin blocks were stained with H&E and examined carefully under the light microscope, independently by two pathologists, one who is experienced in diagnosing HD (observer 2) and another who has limited experience with HD reporting (observer 1). Presence or absence of ganglion cells and hypertrophied nerve bundles were recorded separately for submucosa and muscularis propria as binary variables in ganglionic segment. Similar findings were recorded for aganglionic segments as well. If the results were doubtful or suspicious it was recorded as equivocal. Positive interpretation of Non-Hirschsprung's

disease (NHD) was based on the identification of at least one ganglion cell in a representative serial sections in either submucosal or myenteric plexus. The absence of ganglion cells with or without hypertrophic nerve fibre in submucosa and muscularis propria was considered as HD.

Calretinin IHC interpretation:

All corresponding sections of 68 blocks were stained with Calretinin IHC by above mentioned procedure and evaluated for immunoreactivity by the same two observers blinded both from each other and from the initial H&E diagnosis. Calretinin immunoreactivity and pattern of staining for ganglion cells and nerve fibres in different layers of bowel including lamina propria, muscularis mucosa, submucosa and muscularis propria were evaluated for both ganglionic and aganglionic segments of the IHC stained slides independently by two pathologists. Results were recorded separately for all four layers.

Calretinin was considered as positive if any of the specific findings below were present:

1) Intense, granular and linear staining of nerve fibres in the lamina propria, muscularis mucosa, submucosa and muscularis propria

2) Diffuse strong cytoplasmic and nuclear staining of ganglion cells in the submucosal and/or in the myenteric plexus, and the supporting Schwann cells and nerve cells (excluding Mast cell immunopositivity).

Nonspecific cytoplasmic immunoreactivity was present in sparse ovoid cells (probably mast cells) in the submucosa of all biopsies and was considered as internal positive control. Absence of neural calretinin immunoreactivity in both submucosal or myenteric plexus in aganglionic biopsies was considered diagnostic of HD. However, large nerves in the submucosa which showed axonal immunoreactivity, if present, were recorded separately.

The intensity and pattern of ganglion cells and nerve fibre staining were evaluated semi-quantitatively by light microscopy both in submucosal and myenteric plexuses.

IHC reactivity in ganglion cells was evaluated as follows:

0 – No staining or impossible to visualise ganglion cell,

1 – Mild staining,

2 – Moderate staining,

3 – Strong staining.

In ganglion cells, the staining was evaluated both in nucleus and in cytoplasm. Reactivity for staining of neural plexuses was evaluated by an analogous scale [95].

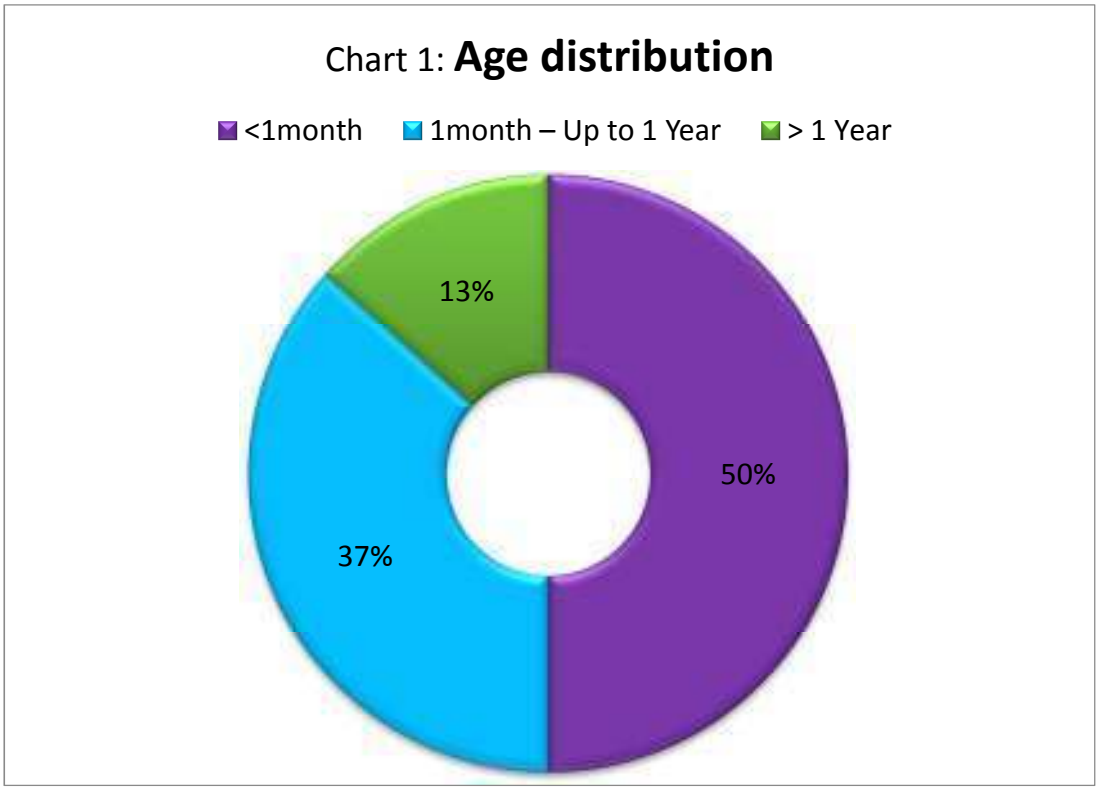
Statistical Analysis

Data analysis was done by SPSS for Windows. Descriptive data were shown as mean ± standard deviation or number of cases and percentages. The data of observer 2 was used to assess diagnostic performance of Calretinin over H&E (e.g. sensitivity, specificity, positive and negative predictive values, and accuracy). We applied Cohen's k coefficient [108] to assess the agreement between the analysis carried out by observer 1 and 2 for both H&E and Calretinin immunohistochemistry. A p value less than 0.05 was considered statistically significant.

OBSERVATIONS AND RESULTS

Table 02: Age distribution of study population

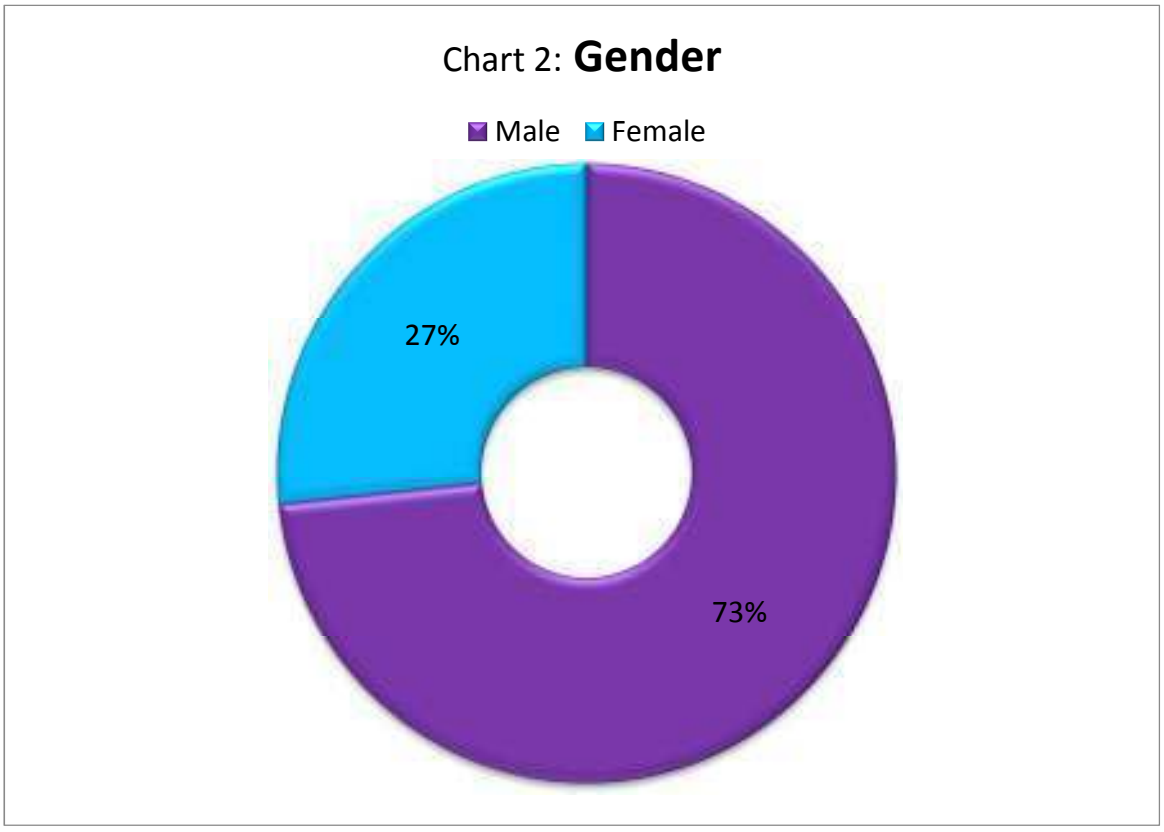
Age	Frequency	Percent (%)
<1month	15	50%
1month – Up to 1 Year	11	36.6%
> 1 Year	4	13.4%
Total	30	100



Most of the patients (50%) presented at less than 1 month of age. Only 13.4% patients were more than 1 years of age.

Table 03: Gender distribution of study population

Gender	Frequency	Percent (%)
Male	22	73.3%
Female	8	26.7%
Total	30	100%

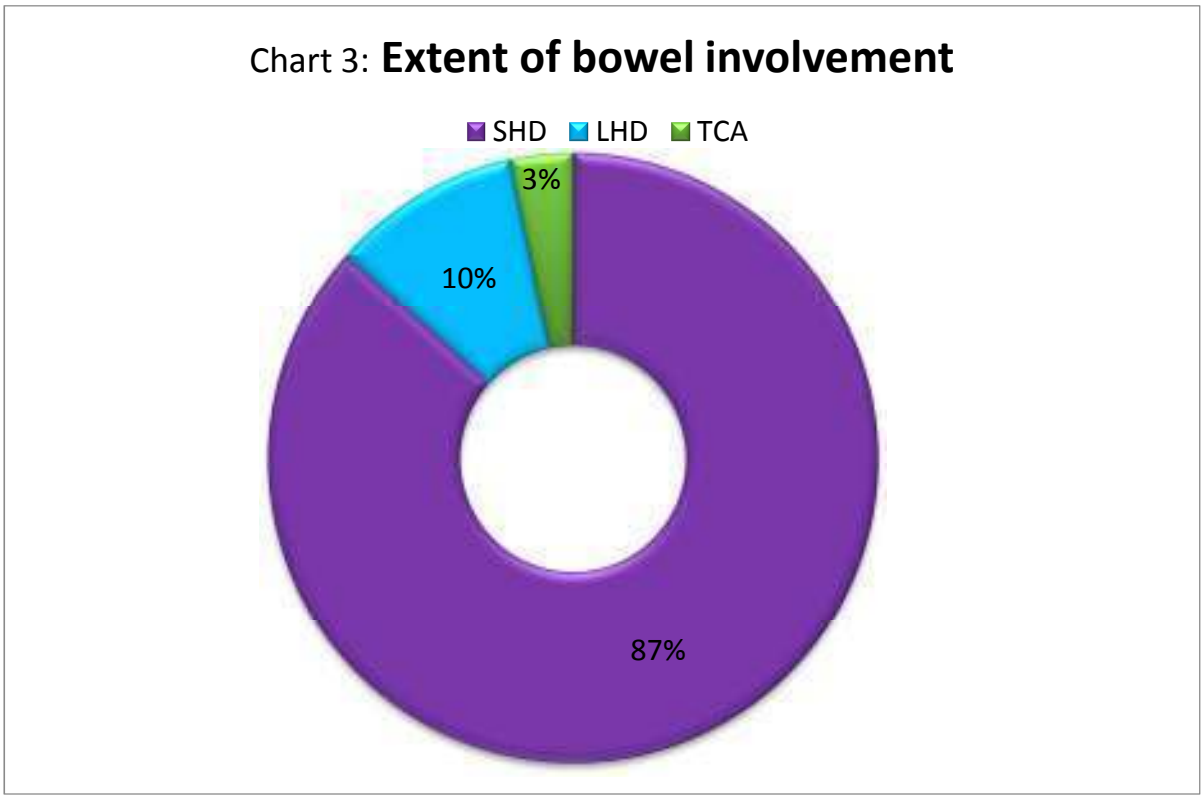


73.3% of the patients presented with clinical diagnosis of HD were males.

Male: female ratio was 2.75:1.

Table 04: Clinical Extent of involvement

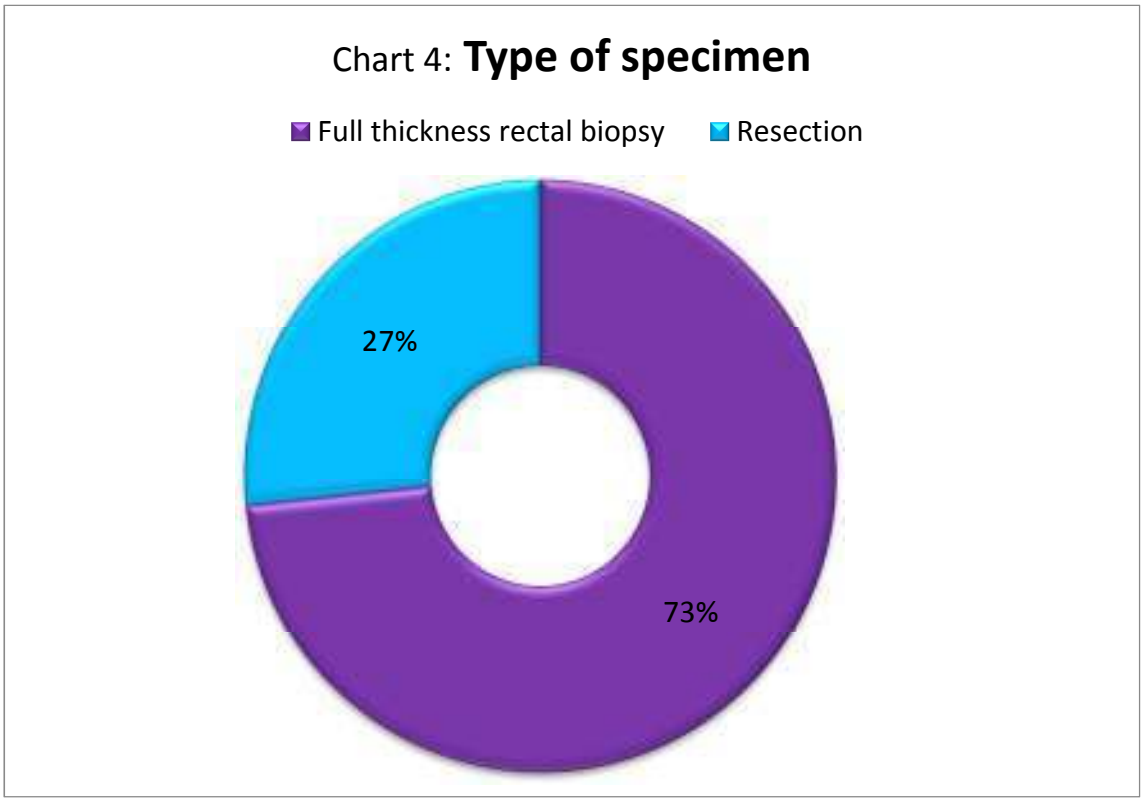
Extent of HD	Frequency	Percent (%)
SHD	26	86.7
LHD	3	10.0
TCA	1	3.3
Total	30	100



Most common form of HD was classic Short segment HD i.e. in 86.7% of cases the disease limited to recto sigmoid portion bowel. Total colonic aganglionosis was seen in one patient (3.3%).

Table 05: Types of specimen

Specimen	Frequency	Percent (%)
Full thickness biopsy	22	73.3
Resection	8	26.7
Total	30	100.0



Most common type of specimen received in our lab for diagnosing HD was full thickness rectal/colon biopsy (73.3%). Eight patients (26.7%) underwent upfront resection.

Table 06: Positive percentage of ganglion cells based on H&E for observer 2

Bowel layers	Aganglionic segment		Ganglionic segment	
	Ganglion cells N (%)	Hypertrophic nerve fibres N (%)	Ganglion cells N (%)	Hypertrophic nerve fibres N (%)
Submucosa	4 (13.3%)	19 (63.3%)	26 (89.6%) $	3 (10.3%) $
Muscularis propria	5 (18.5%)*	19 (70.3%)*	24 (85.7%) #	3 (7.1%) #

*n=27, $ n=29, # n=28 – The differences in number of samples is due to the unavailability of that particular layer in the sample. N=30 in all others.

Submucosa and Muscularis propria showed ganglion cells in 4 and 5 cases respectively. The presence of ganglion in 5 cases resulted in diagnosis of Non-HD. Hypertrophic nerve fibres were seen in around 65-70% of cases in the study population.

In clinical ganglionic segment, ganglion cells could not be identified in around 13% of cases resulting in sensitivity of 87% for H&E in identifying ganglion cells.

When five patients with ganglion cells (patients confirmed with NHD) were excluded, 19 of 25 (76%) patients and 19 of 22 (as muscularis propria was absent in three samples) (86.4%) patients showed hypertrophic nerve bundles in submucosa and muscularis propria respectively.

Table 6A: Sensitivity, Specificity and PPV for Nerve bundle hypertrophy in diagnosis of HD.

	HD diagnosed by absence of ganglion cells		**Total**
Hypertrophic nerve bundles	Positive	Negative	
Positive	19	0	**19**
Negative	3	5	**8**
Total	**22**	**5**	**27**

Hypertrophic nerve bundles were absent in 3 of 5 patients diagnosed as HD by H&E in both layers. The table for sensitivity, specificity and PPV calculation for nerve bundle hypertrophy in diagnosing HD is shown above. The sensitivity, specificity and PPV were 86.4%, 100% and 100% respectively.

Table 07: Hypertrophic nerve fibres in aganglionic segment comparison between Short segment HD vs Long segment HD based on H&E for observer 2

Bowel layers	**Short segment n=26** **N (%)**	**Long segment n=4** **N (%)**
Submucosa	18 (69.2%)	2 (50%)
Muscularis propria	17 (73.9%)*	2 (50%)

*number of samples was 23 (n= 23) as Muscularis propria was not included in the biopsy of three patients.

Hypertrophic nerve fibres were seen in 50% of patients (2/4) with long segment involvement and in around 70% of patients with suspected short segment involvement. However, when 5 cases of NHD were excluded, submucosa and muscularis propria showed nerve hypertrophy in 17 of 21 and 16 of 18 cases respectively. In short, Hypertrophic nerve bundles were present in 84.6% of confirmed short segment HD diagnosed by H&E.

Table 08: Calretinin IHC expression in both aganglionic and ganglionic segments for observer 2

Bowel layers	Aganglionic segment		Ganglionic segment	
	Ganglion cells	Intrinsic nerve fibres	Ganglion cells	Intrinsic nerve fibres
Lamina Propria	NA	5(17.2%) $	NA	24(82.8%) $
Muscularis mucosa	NA	4 (13.8%) $	NA	26(89.7%) $
Submucosa	5 (16.7%)	8 (26.7%)	26(89.7%) $	26(89.7%) $
Muscularis propria	5(18.5%)*	8 (29.6%)*	24(85.7%) #	24(82.8%) #

*n=27, $ n=29, # n=28 - The differences in number of samples is due to the unavailability of that particular layer in the sample. N=30 in all others.

Calretinin expression was seen in 5 cases resulting in diagnosis of NHD similar to H&E (Table 6). Of 25 cases diagnosed as HD by ganglion cell negativity with IHC, three cases (12% cases) showed immunoreactivity in nerve fibres. All five cases with Calretinin immunoreactivity in ganglion cells showed immunoreactivity in nerve fibres as well. When nerve fibre

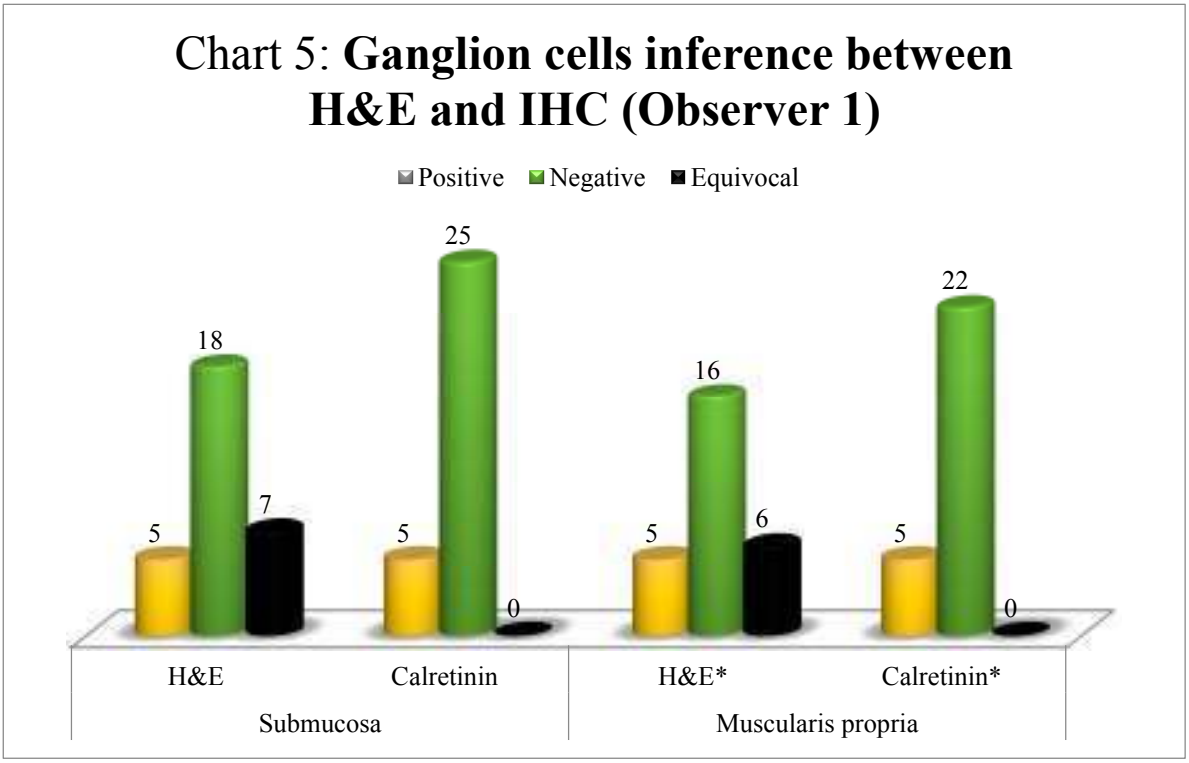
immunoreactivity was taken as the criteria for diagnosis, this method had a sensitivity and specificity 88% and 100% respectively.

In clinical ganglionic segment, ganglion cells could not be identified in around 13% of cases resulting in sensitivity of 87% for H&E in identifying ganglion cells.

Table 09: Ganglion cell inference between H&E and Calretinin IHC for Observer-1 in aganglionic segment.

Ganglion cells in aganglionic segment	Submucosa		Muscularis propria	
	H&E	Calretinin	H&E*	Calretinin*
Positive	5 (16.7%)	5 (16.7%)	5 (18.5%)	5 (18.5%)
Negative	18 (60%)	25 (83.3%)	16 (59.2%)	22 (81.5%)
Equivocal	7 (23.3%)	0	6 (22.2%)	0

*n=27- The differences in number of samples is due to the unavailability of that particular layer in the sample. N=30 in all others.

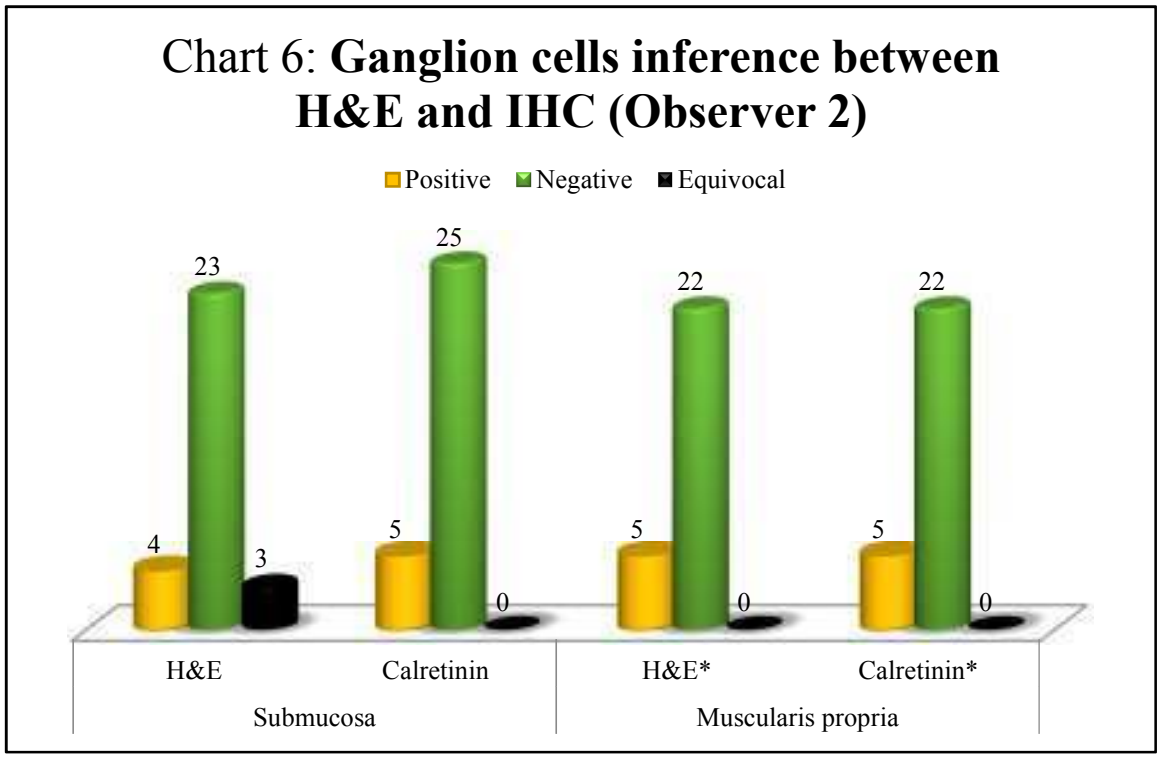


Observer 1, with limited experience, recorded equivocal findings (i.e. suspicious of ganglion cells) in around 23% using H&E. However, Calretinin IHC could provide a definite answer to this suspicion in all the cases. There was no equivocal reading in any of the samples using Calretinin IHC. The sensitivity and positive predictive value (PPV) of H&E are both 100%.

Table 10: Ganglion cell inference between H&E and Calretinin IHC for Observer-2 in aganglionic segment.

Ganglion cells in aganglionic segment	Submucosa		Muscularis propria	
	H&E	Calretinin	H&E*	Calretinin*
Positive	4 (13.3%)	5 (16.7%)	5 (18.5%)	5 (18.5%)
Negative	23 (76.7%)	25 (83.3%)	22 (81.5%)	22 (81.5%)
Equivocal	3 (10%)	0	0	0

*n=27- The differences in number of samples is due to the unavailability of that particular layer in the sample. N=30 in all others.



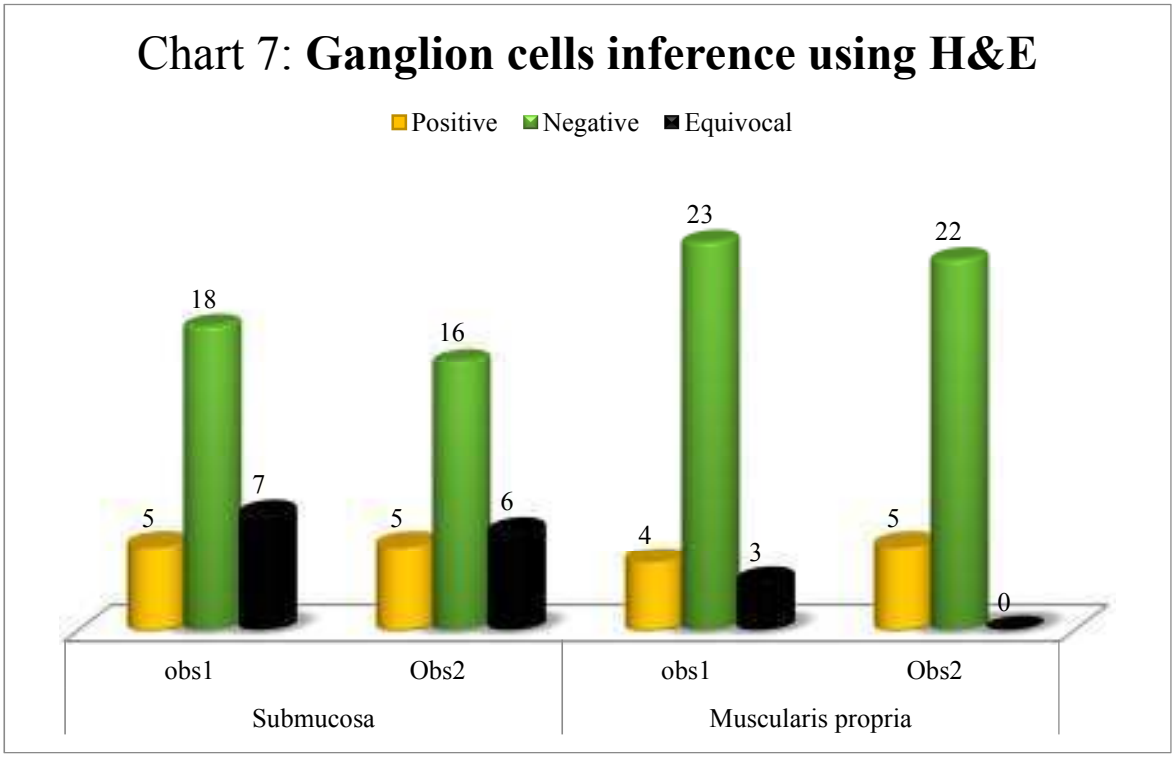
In submucosal layer, by H&E, four samples showed ganglion cells in the aganglionic segment and three patients were recorded as suspicious for the presence of ganglion. Subsequently, Calretinin IHC showed positivity for ganglion cells in one additional patient recorded as negative by H&E. All the suspicious cases by H&E stained negative with IHC. There was no discrepancy in the detection of ganglion cells in muscularis propria. There was no equivocal reading with IHC in both observers.

The sensitivity and PPV for H&E in our study are 90% and 100% respectively.

Table 11: Inter observer variation in detecting ganglion cell in the aganglionic segment based on H&E.

Aganglionic segment	Observer 1		Observer 2	
	Submucosa	Muscularis propria*	Submucosa	Muscularis propria*
Positive	5 (16.7%)	5 (18.5%)	4 (13.3%)	5 (18.5%)
Negative	18 (60%)	16 (59.2%)	23 (76.7%)	22 (81.5%)
Equivocal	7 (23.3%)	6 (22.2%)	3 (10%)	0

*n=27- The differences in number of samples is due to the unavailability of that particular layer in the sample. N=30 in all others.



The positive identification of ganglion did not differ significantly between the two observers (10 vs 9 cases) using H&E. However, the number of equivocal identifications of ganglion was higher in Observer 1 (21.6%) compared to observer 2 (5%).

Concordance of independent observational data by the two observers is shown below:

Table 11 A: Concordance-Discordance table in detecting ganglion cell in aganglionic segment based on H&E between two observers.

	Observer 1				**Total**
Observer 2		*Positive*	*Negative*	*Equivocal*	
	Positive	7	2	1	**10**
	Negative	0	39	1	**40**
	Equivocal	2	4	1	**7**
Total		**9**	**45**	**3**	**57**

There were 57 observations for each observer for detection of ganglion cells- 30 in submucosa and 27 in muscularis propria. Both observers were blinded from each other and the data was analysed for inter observer concordance. The kappa value was interpreted as follows:

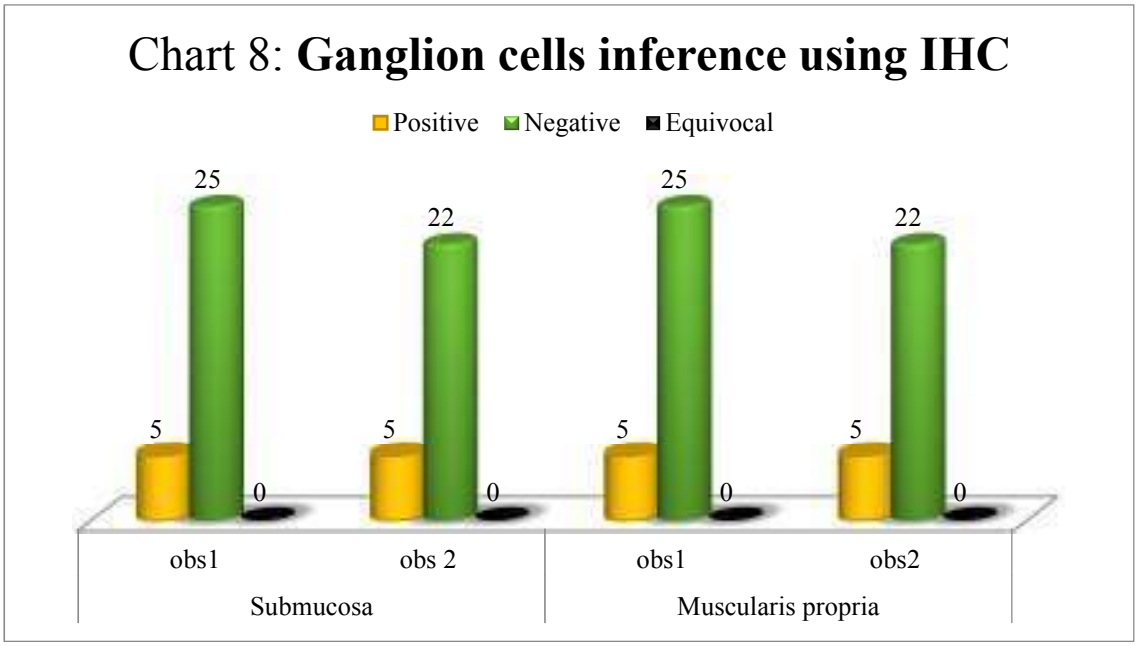
- Poor agreement = Less than 0.20
- Fair agreement = 0.20 to 0.40
- Moderate agreement = 0.40 to 0.60
- Good agreement = 0.60 to 0.80
- Very good agreement = 0.80 to 1.00

In our data, there were 47 agreements and Cohen's kappa co-efficient was 0.574 (95% CI 0.36- 0.78). The agreement was moderate.

Table 12: Inter observer inference for ganglion cell in the aganglionic segment based on Calretinin IHC.

Aganglionic segment	Observer 1		Observer 2	
	Submucosa	Muscularis propria*	Submucosa	Muscularis propria*
Positive	5 (16.7%)	5 (18.5%)	5 (16.7%)	5 (18.5%)
Negative	25 (83.3%)	22 (81.5%)	25 (83.3%)	22 (81.5%)
Equivocal	0	0	0	0

*n=27- The differences in number of samples is due to the unavailability of that particular layer in the sample. N=30 in all others.

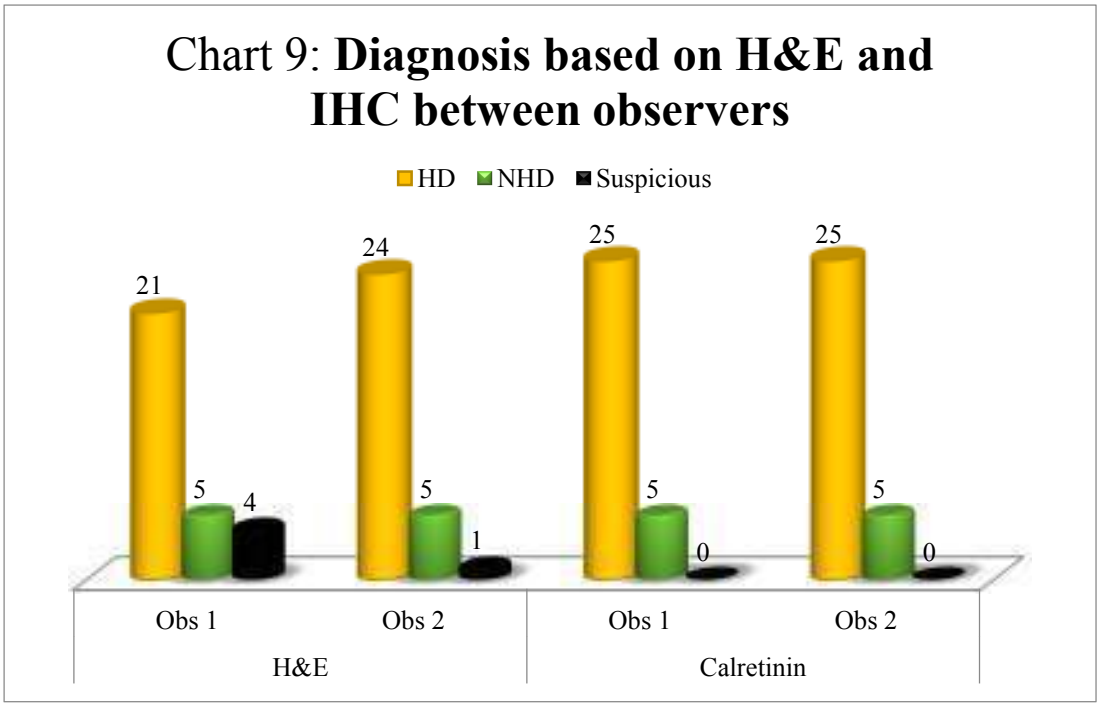


There was no discordance in the indentification of ganglion cells between both observers with agreement in all 57 observations resulting in Cohen's kappa of 1.00.

Based on their observations, both observers were required to provide the final diagnosis independently. The diagnosis frequency is as below:

Table 13: Frequency of HD and Non HD based on H&E and Calretinin IHC between two observers.

Number of cases	Initial diagnosis (H&E)		IHC diagnosis (Calretinin)	
	Observer 1	Observer 2	Observer 1	Observer 2
HD	21 (70%)	24(80%)	25 (83.3%)	25 (83.3%)
NHD	5 (16.7%)	5 (16.7%)	5 (16.7%)	5 (16.7%)
Suspicious	4 (13.3%)	1 (3.3%)	0	0



Of the 30 samples received, 70-80% cases were diagnosed as HD by H&E, while 83.3% cases could be diagnosed using Calretinin IHC. This shows that around 13.3 % additional cases could be identified using Calretinin over H&E, improving the diagnostic yield. By H&E there were 4 doubtful diagnosis of HD which could be confirmed using Calretinin IHC thus improving the accuracy.

Table 14: Calretinin Immunoreactivity Index for ganglion cells in the ganglionic segment.

Calretinin Reactivity Index in Ganglionic segment	Numbers N=26 (%) *
2+	7 (27%)
3+	19 (73%)

*In four cases, samples from clinical ganglionic segment showed no ganglion cells; hence excluded from calculation of Calretinin Immunoreactivity Index.

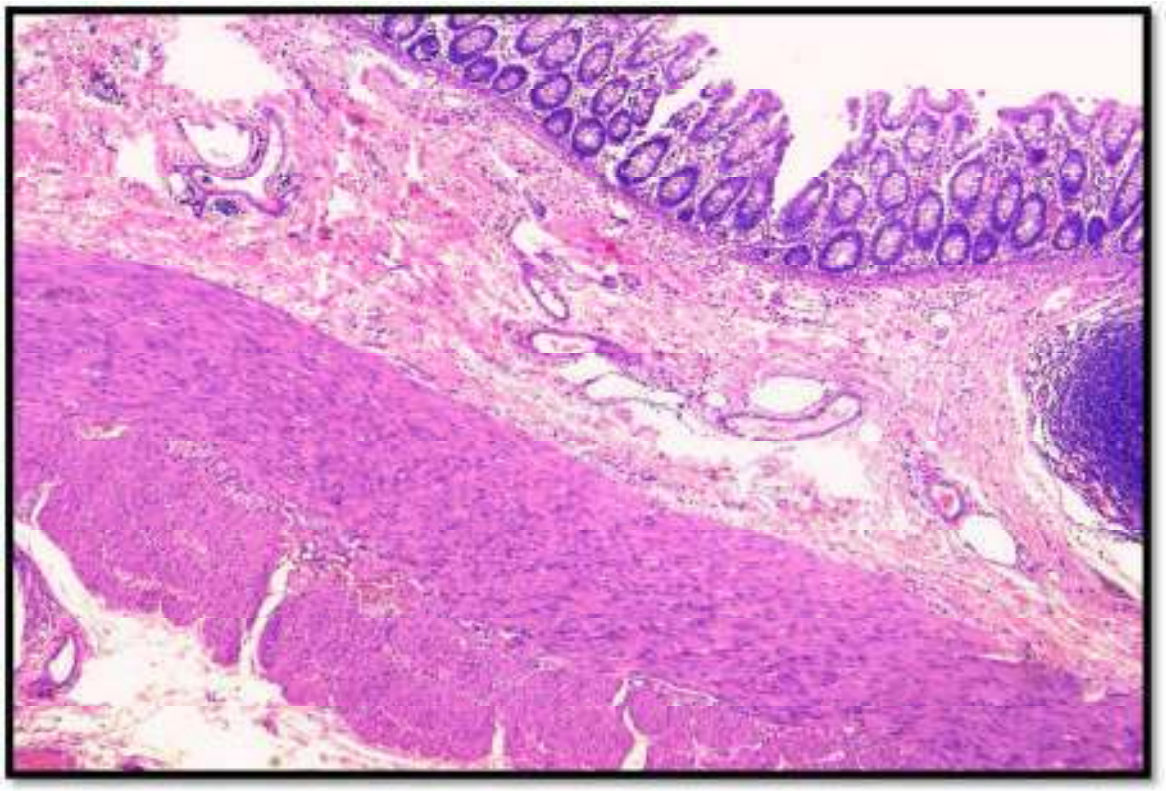


Figure 5: H&E Section shows normal layers of the rectal biopsy (40X)

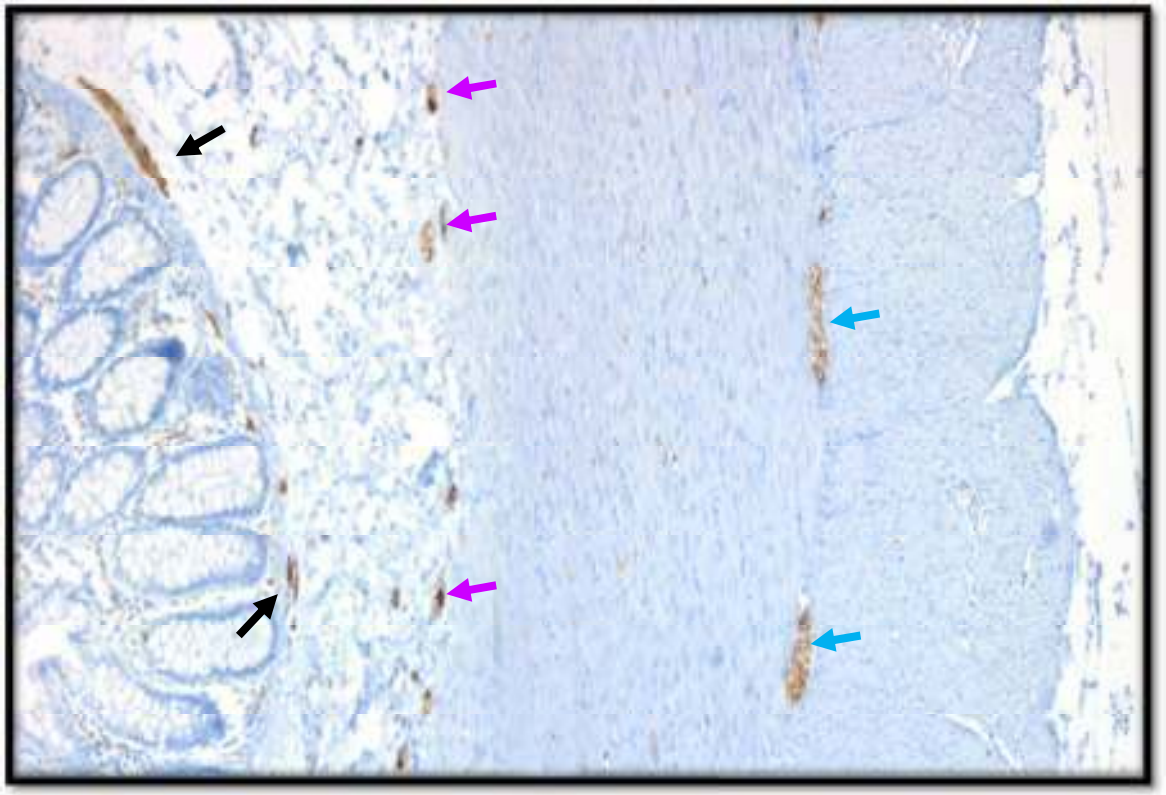


Figure 6: Calretinin IHC section shows pattern of staining in the normal bowel mucosa and to show Meissner's plexus, Henle's plexus and Auerbach's plexus (Black, Pink, Blue arrows respectively) (40X).

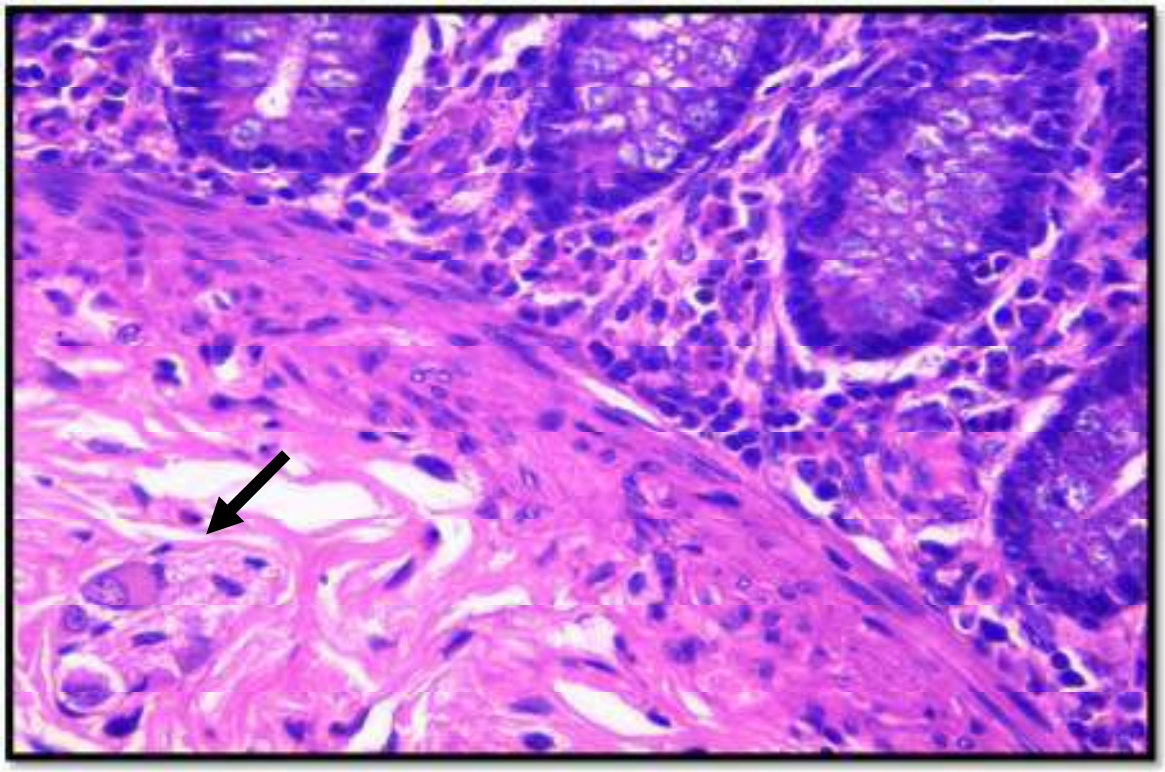


Figure 7: H&E section shows submucosal – Meissner's Plexus (arrow) (400X)

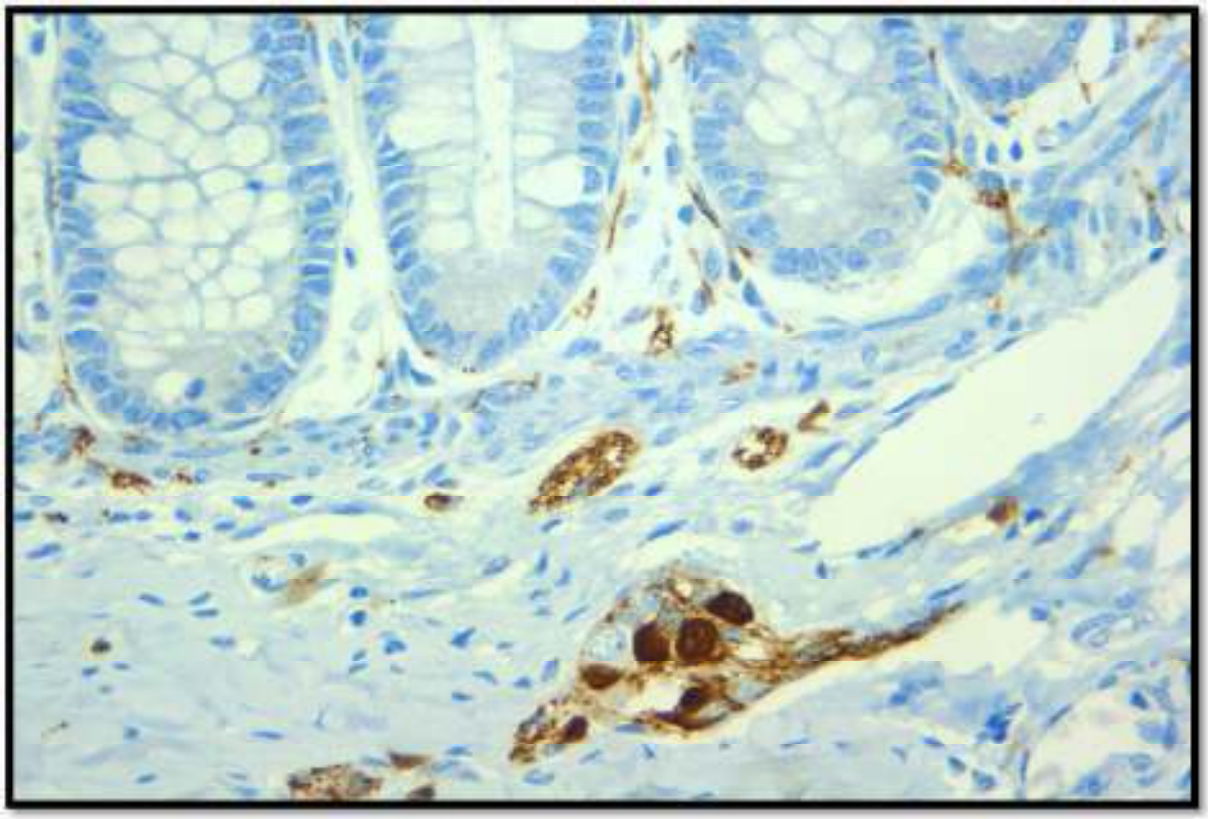


Figure 8: Calretinin IHC section shows immunoreactivity in the submucosal plexus (400X).

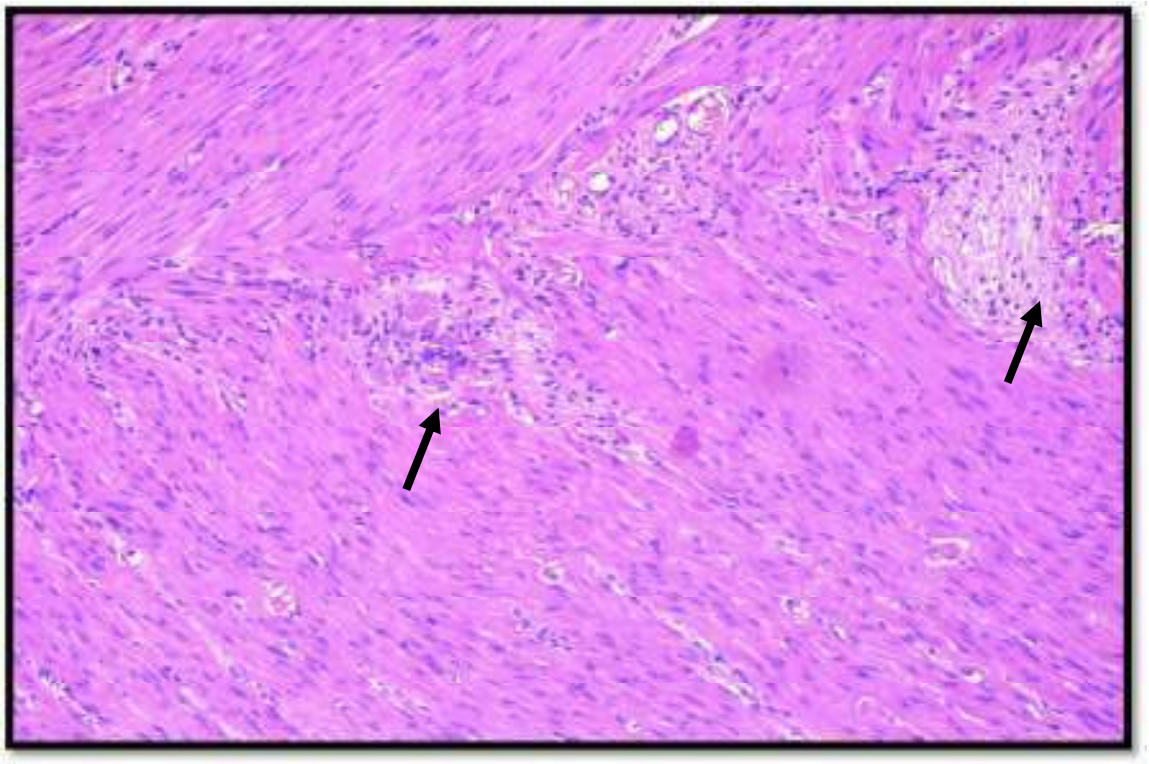


Figure 9: H&E section shows location of Auerbach's plexus between circular and longitudinal muscle layer (arrow) (100X)

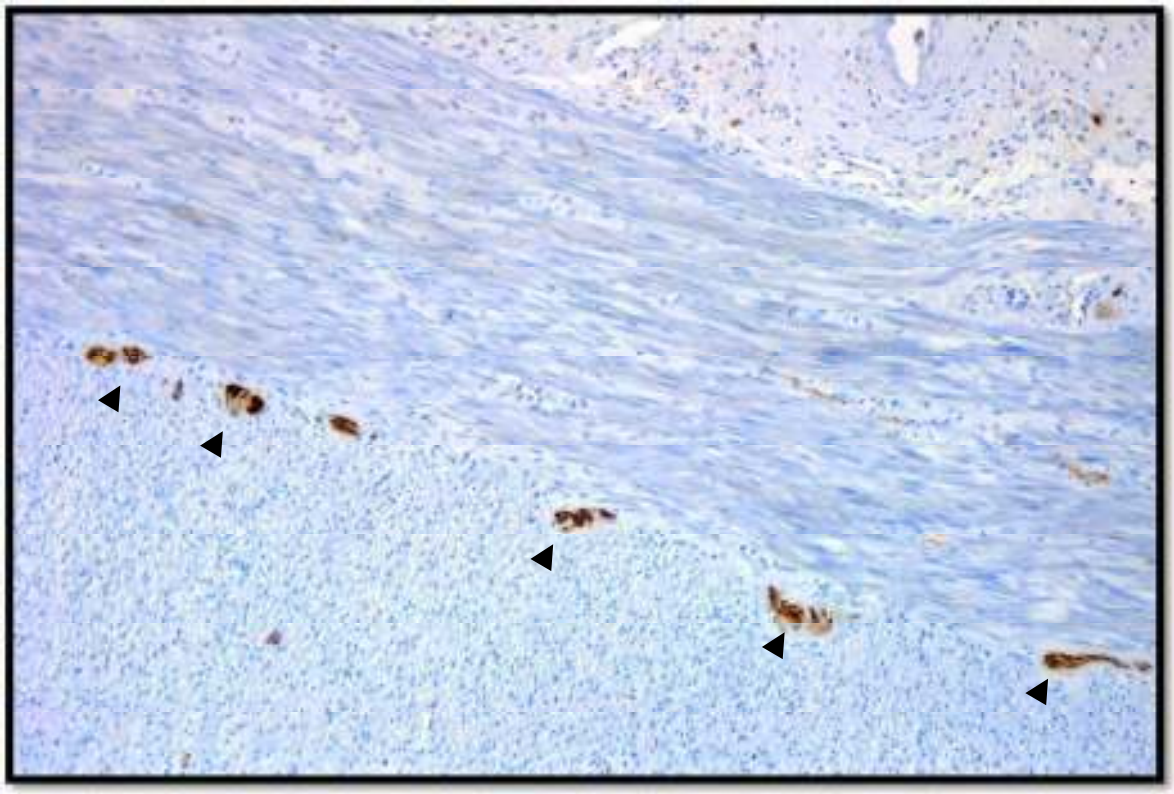


Figure 10: Calretinin IHC section shows immunoreactivity of myenteric ganglia which located at equi-distance for every 1mm of normal rectal mucosa (arrowhead) (100X).

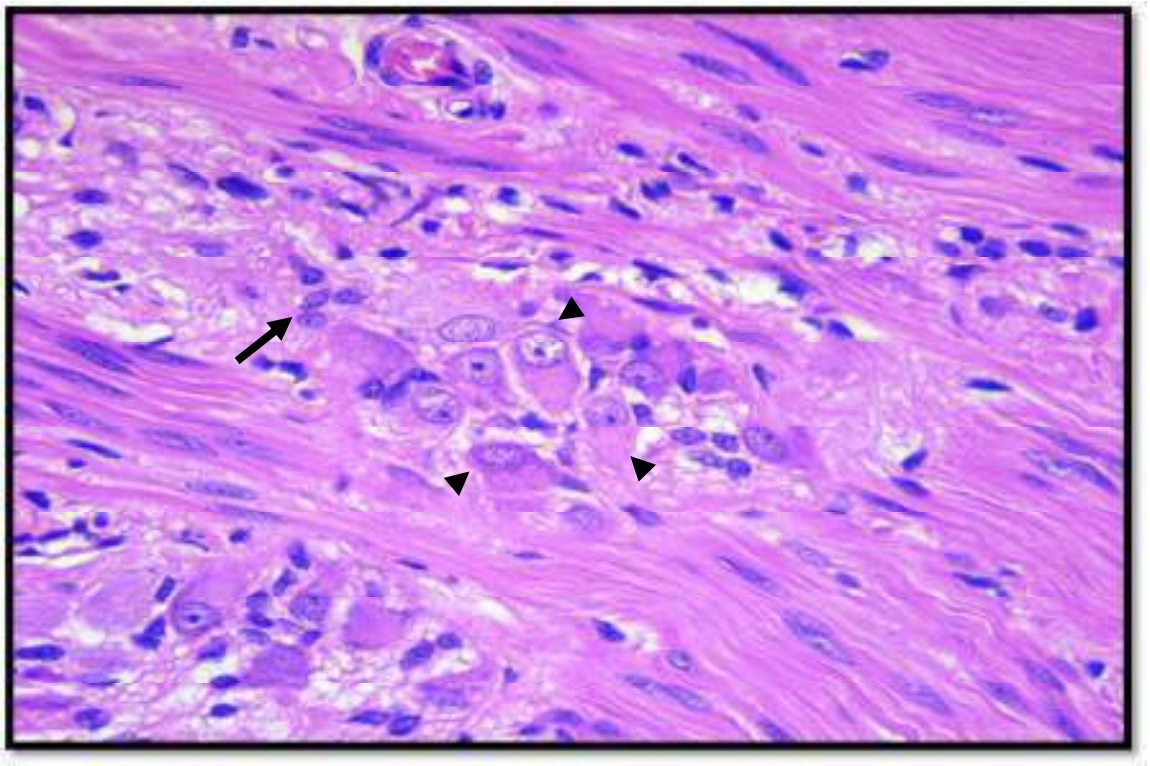


Figure 11: H&E section shows Ganglion cells (neuronal cell bodies) characterized by a large polygonal cells with abundant pink cytoplasm and an eccentric nucleus, prominent nucleoli (arrowhead); spindled neural projections and Schwann cells(arrow) are also intermixed (400X)

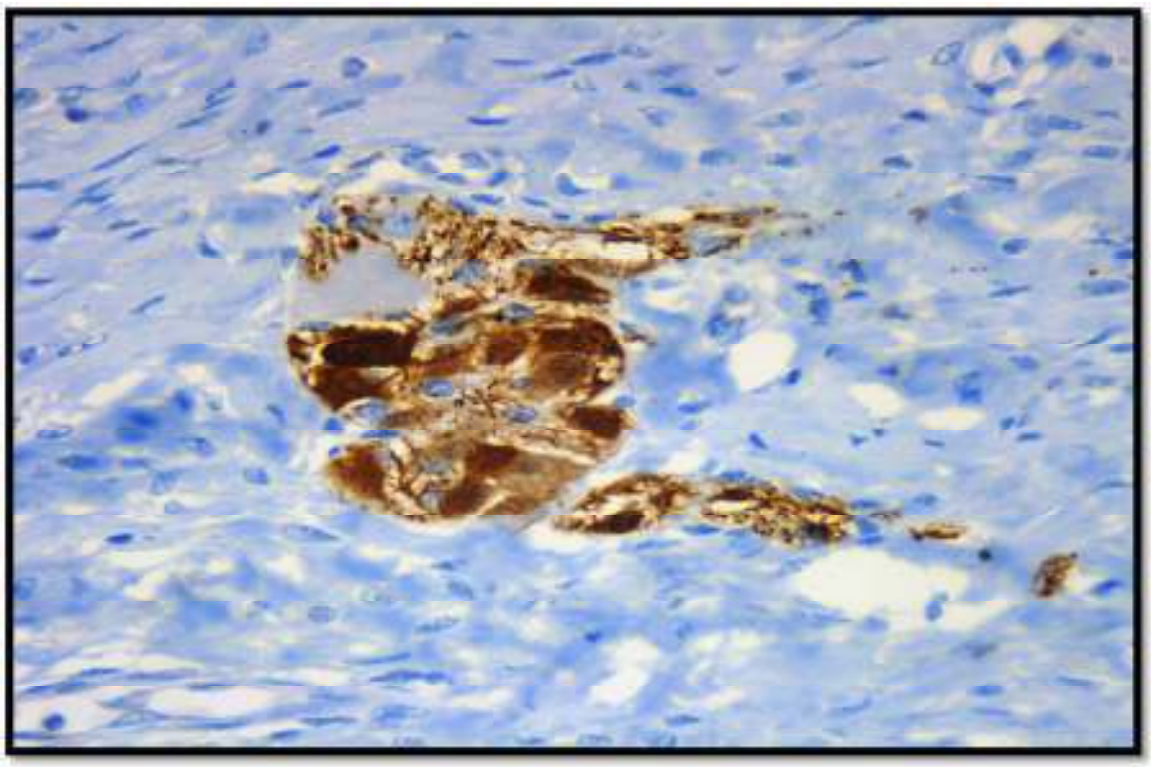


Figure 12: Calretinin IHC section shows strong reactivity to ganglion cells and moderate reactivity to nerve plexus (400X).

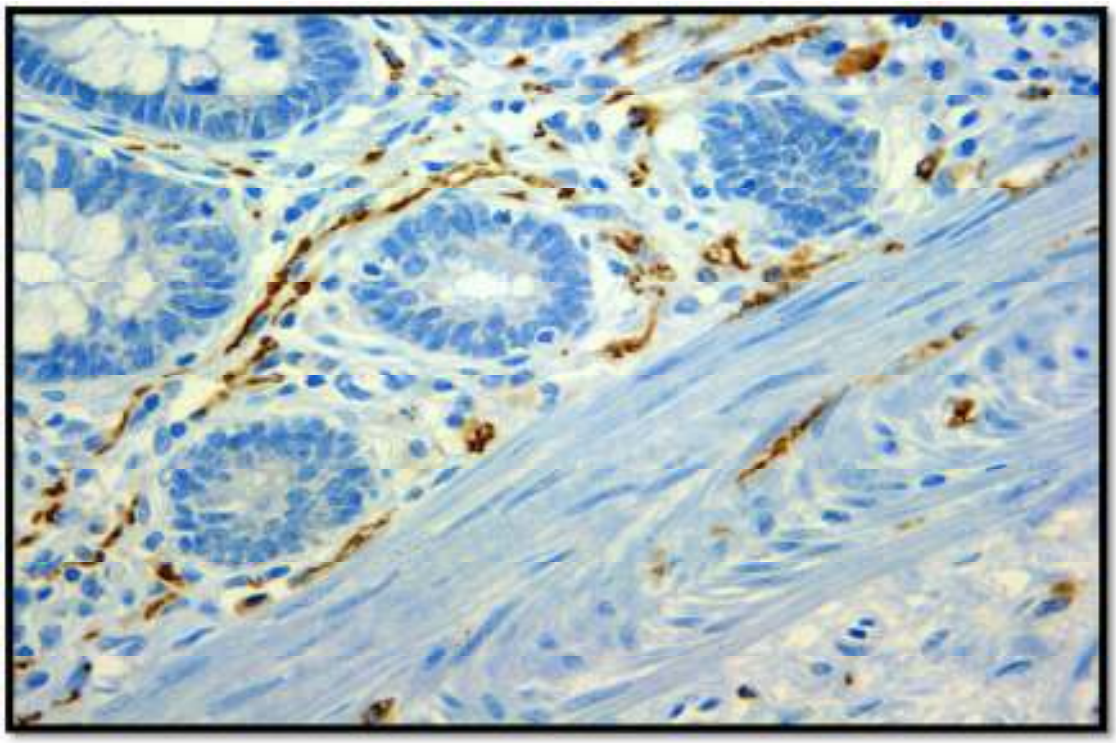


Figure 13: Calretinin IHC section shows lamina propria and muscularis mucosa of rectal biopsy with immunopositive thin, linear, granular nerve fibrils, in a non-HD case (400X).

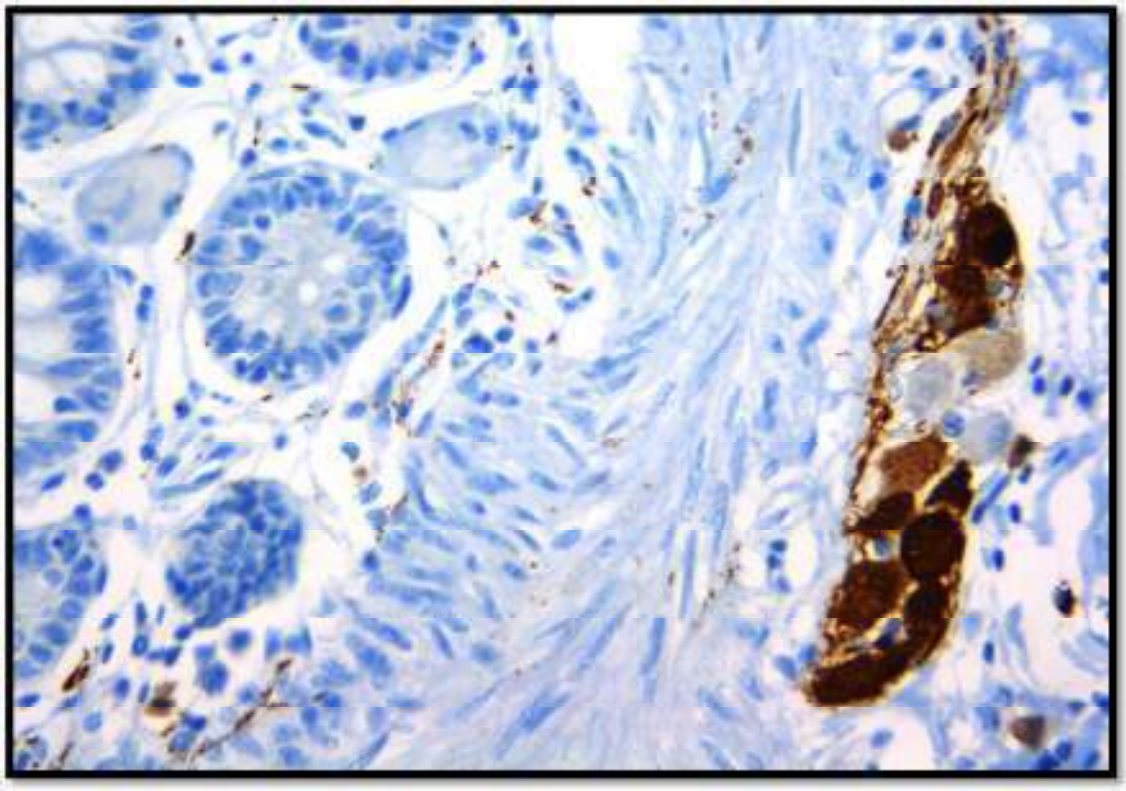


Figure 14: Calretinin IHC section shows diffuse strong (3+) cytoplasmic and nuclear staining in the submucosal ganglion cells (400X).

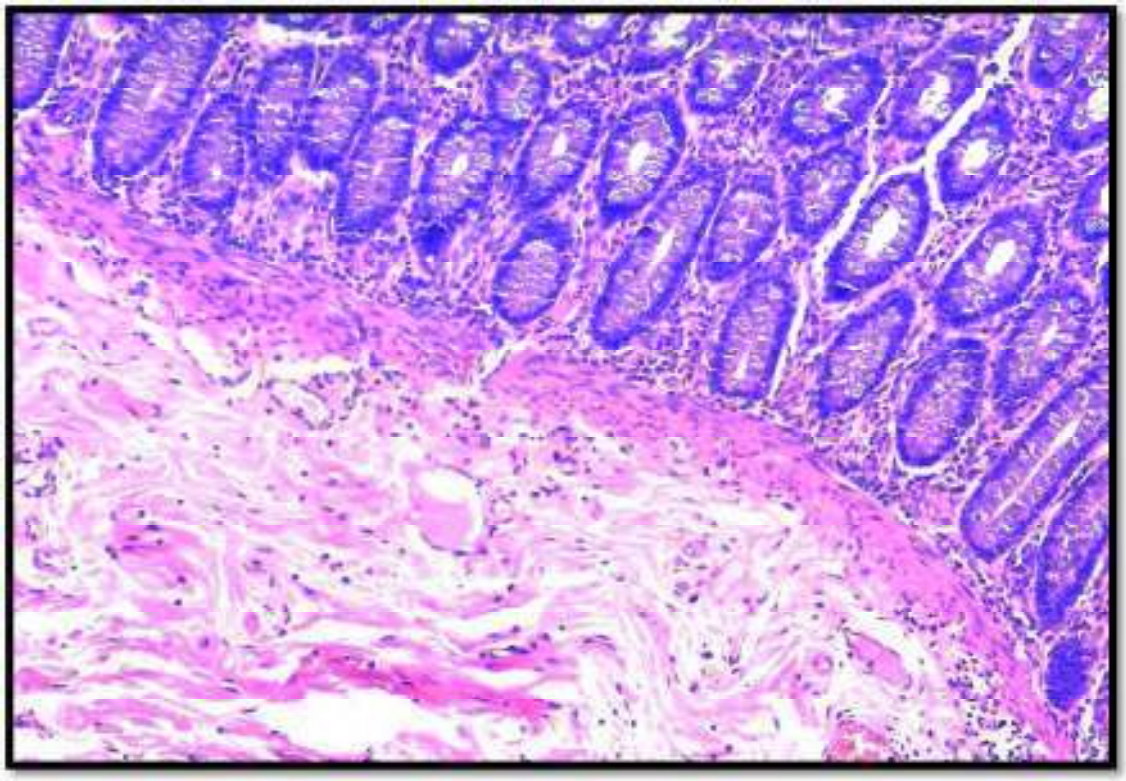


Figure 15: H&E section shows lack of ganglia in the submucosal plexuses in a case HD (100X).

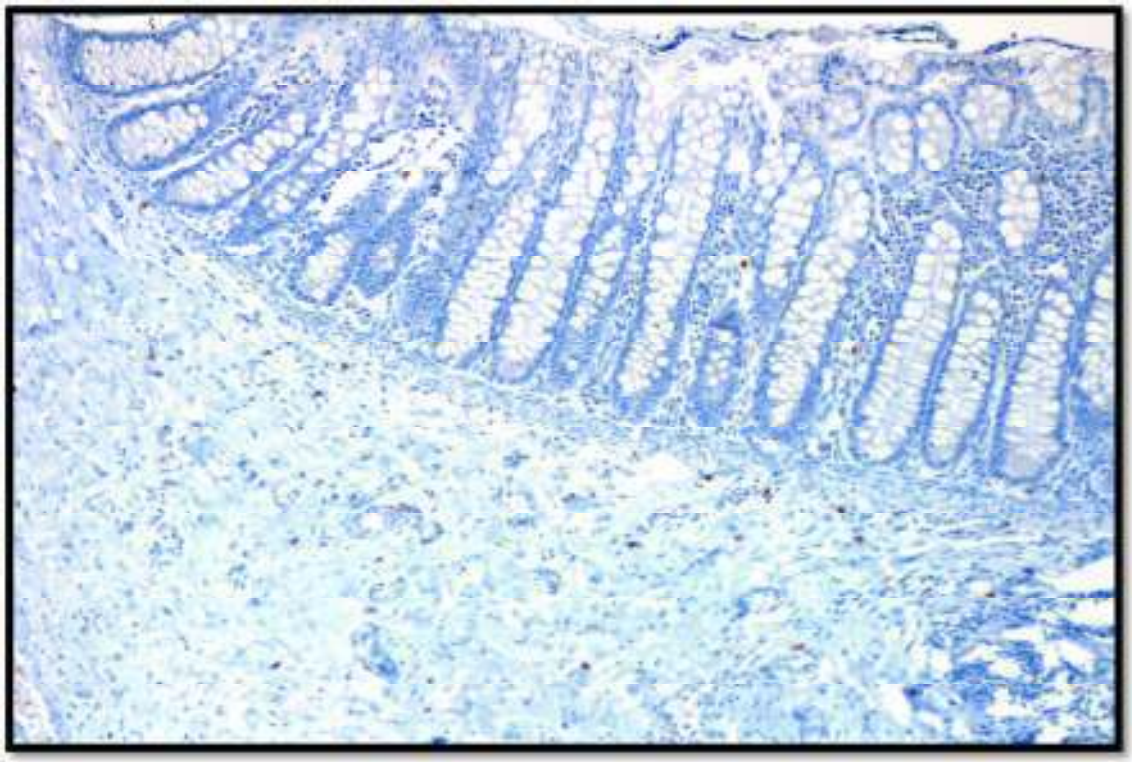


Figure 16: Calretinin IHC section shows total absence of staining either in the submucosa, muscularis mucosa or lamina propria except for mast cells (40X).

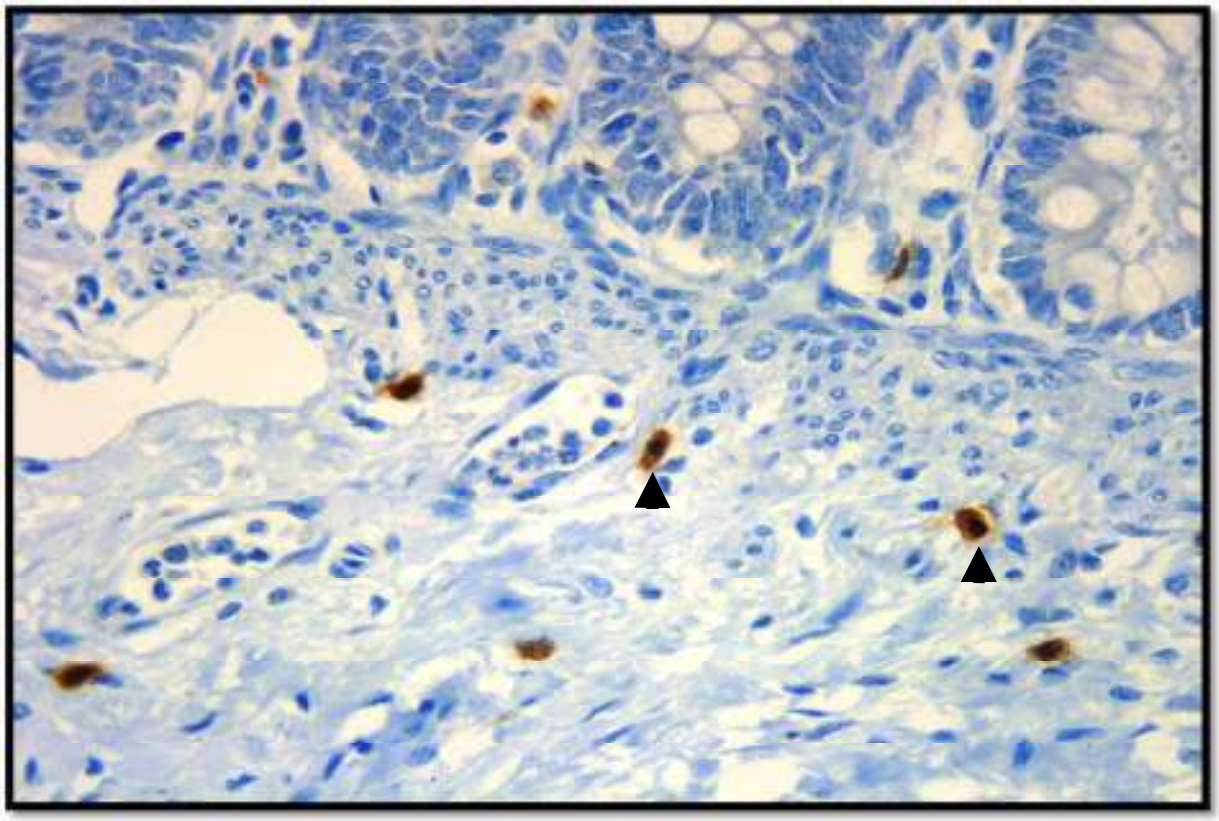


Figure 17: Calretinin IHC section shows some background staining in the HD case, non-neuronal sparse ovoid cells (arrowhead) probable mast cells (400X).

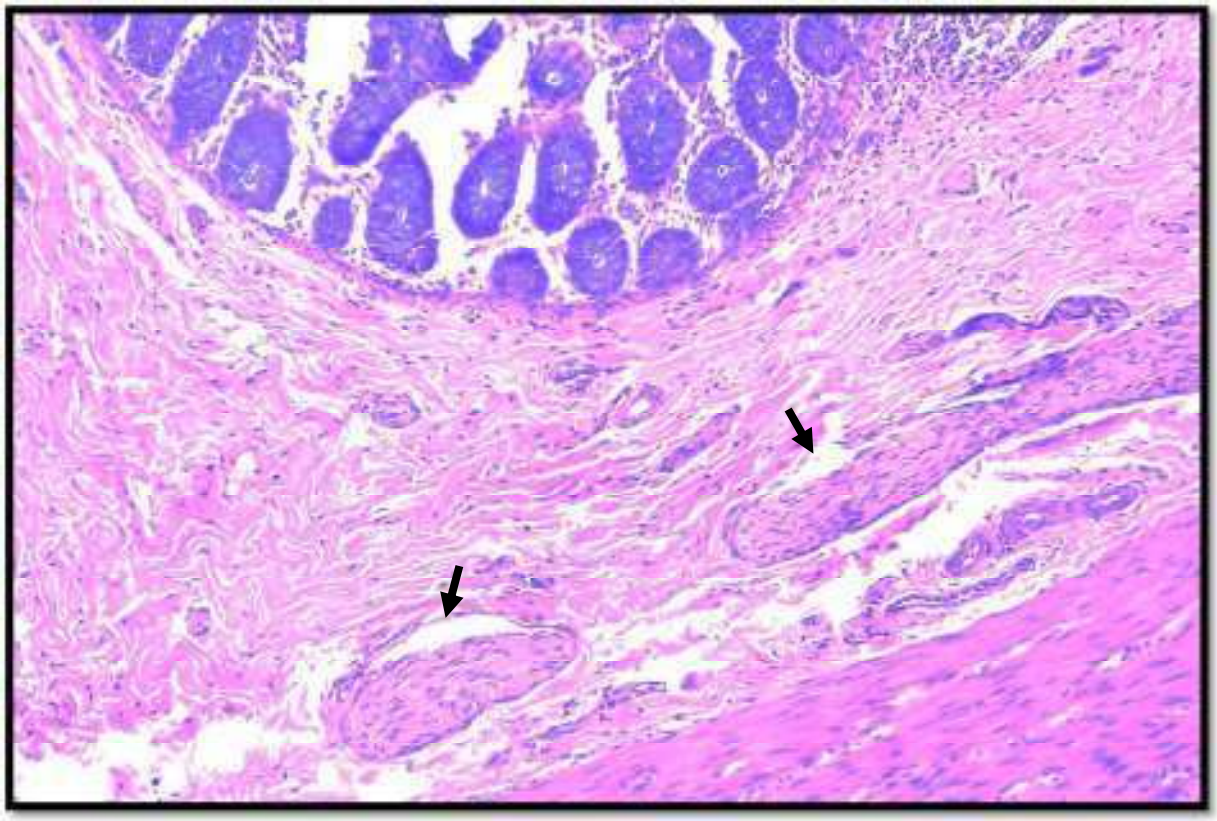


Figure 18: H&E section shows hypertrophic nerve bundles in the submucosa of a HD case (arrow) (100X).

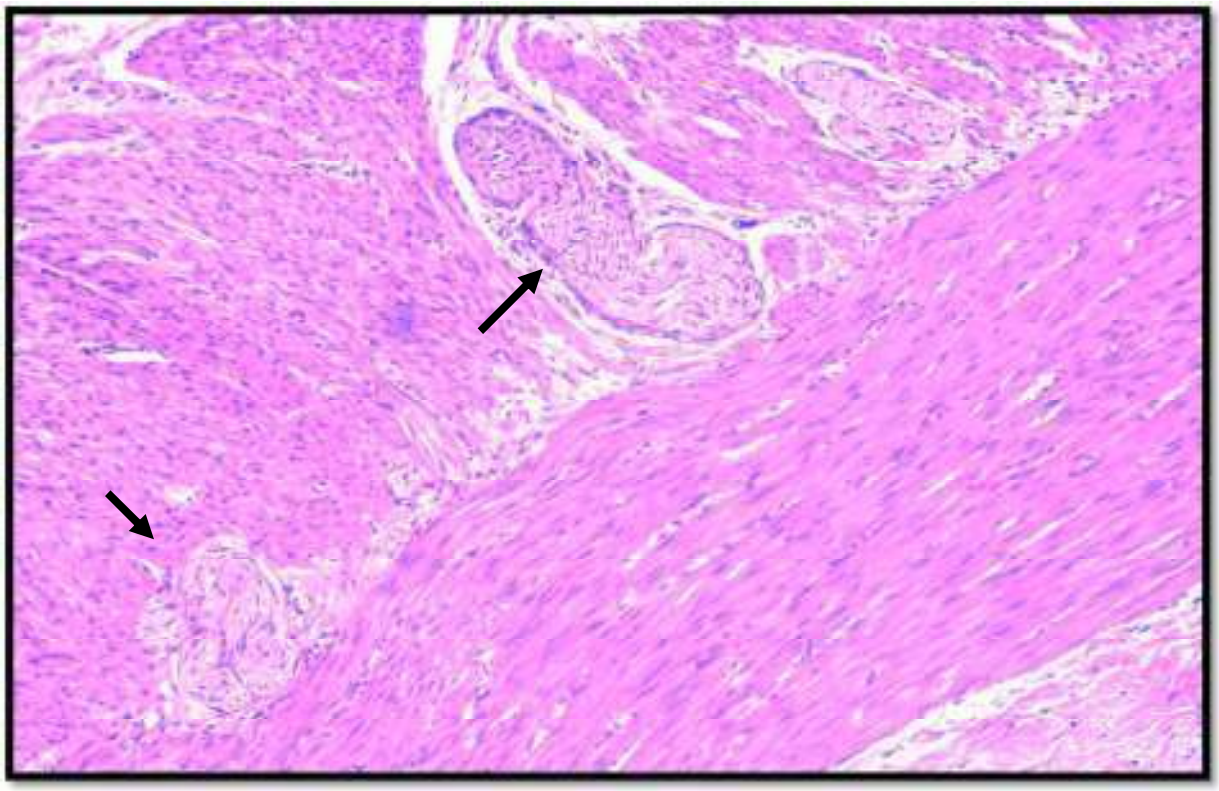


Figure 19: H&E section from a HD case shows Hypertrophic myenteric nerve plexuses (arrows) lacking ganglion cells (100X).

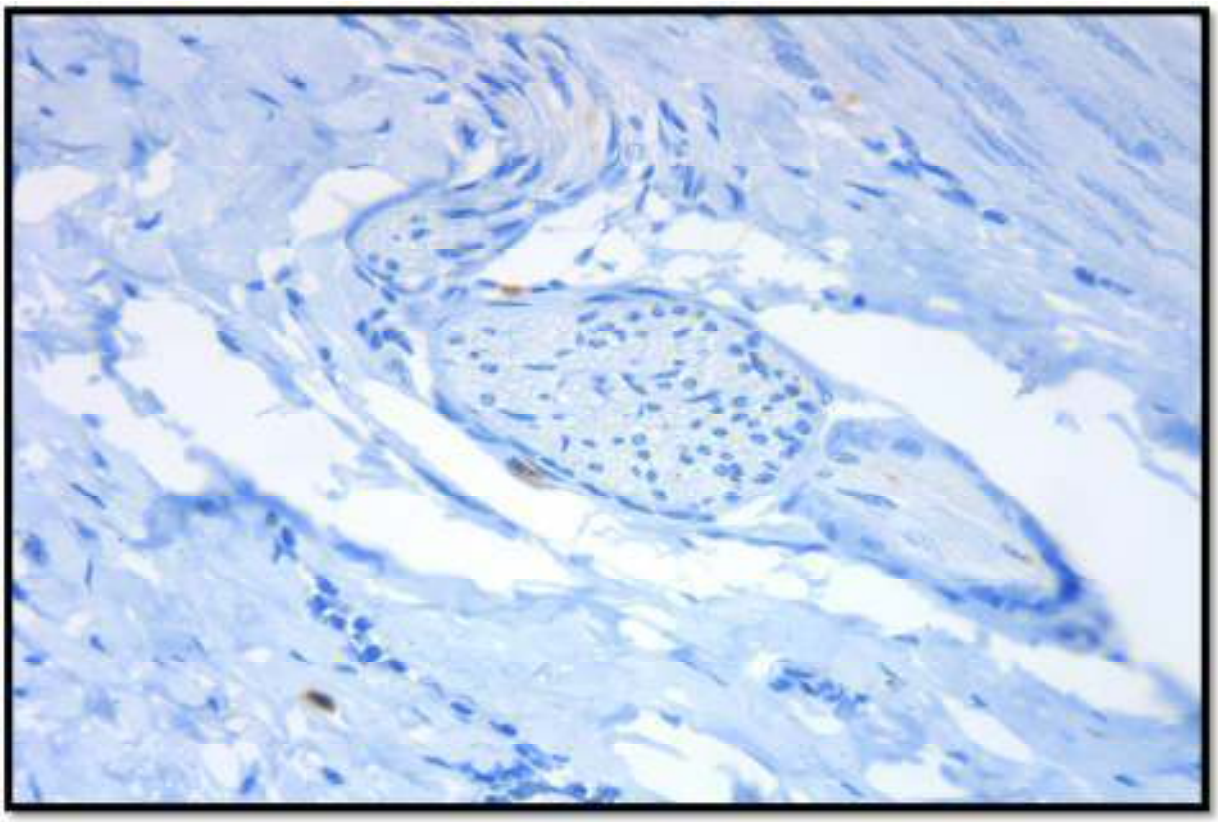


Figure 20: Calretinin IHC section from the HD case shows submucosal hypertrophic nerve trunk with no immunoreactivity for ganglion cells and nerve fibre (400X).

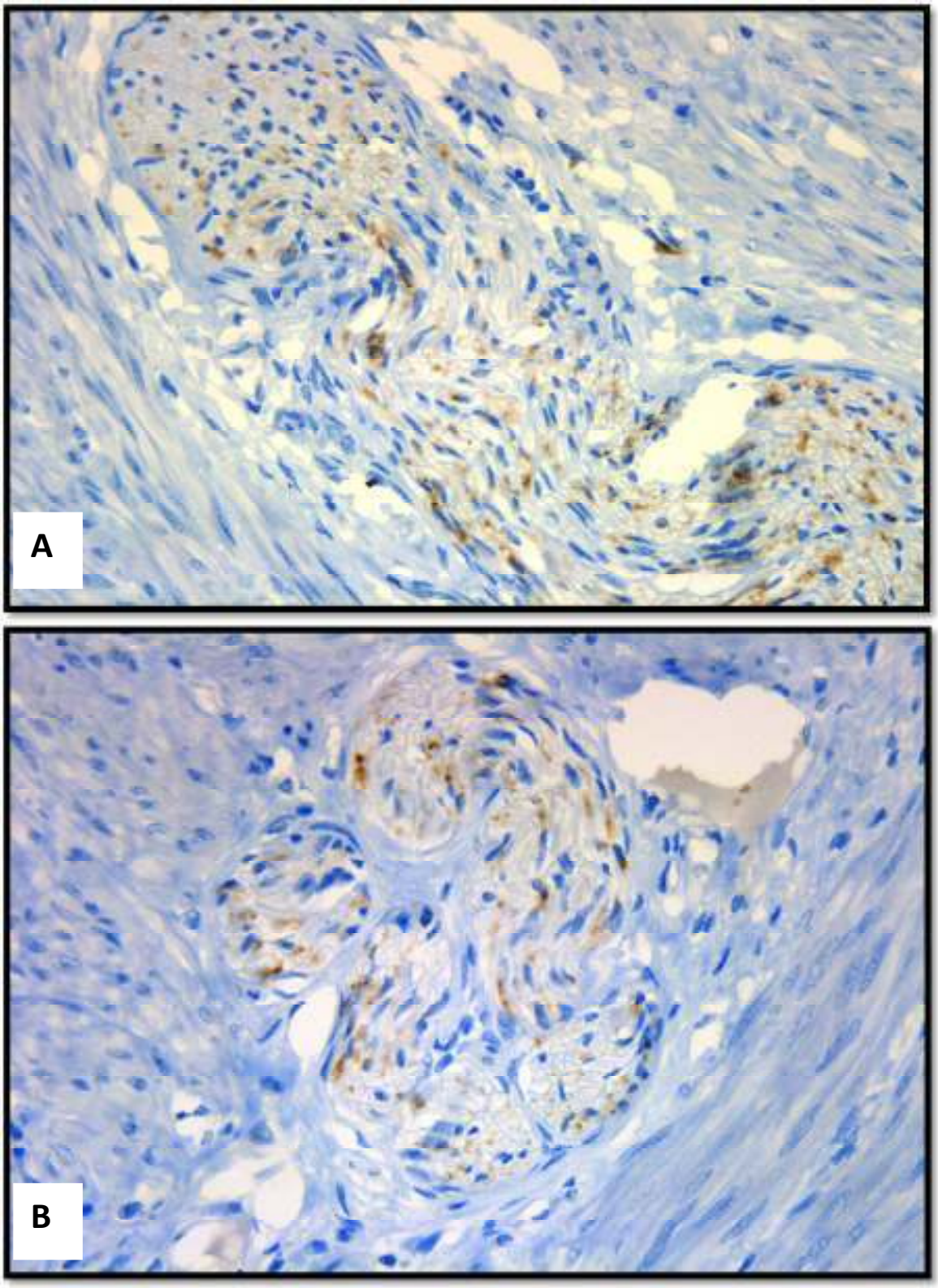


Figure 21: A&B Calretinin IHC sections from the aganglionic segment of a HD case shows faint, punctate (rather than confluent) immunoreactivity in a large calibre myenteric nerve trunk (400X).

DISCUSSION

Our study was done on specimens of 30 suspected cases of Hirschsprung's disease. Patient age ranged from 2 days to 10 years. The median age of presentation was 36 days (Inter quartile range between 6 days and 1year). Of the 30 cases fifteen patients (50%) presented at less than 1 month of age, eleven (36.6%) patients between 1 month and 1 year of age and four (13.4%) patients after the age of 1year. Kannaiyan L et al [105] in their study had twenty three patients (63.8%) at less than 1 month of age. Seven (19.5%) patients presented between 1 month and 1 year of age. Six (16.7%) patients presented after the age of 1.

There were 22 boys and 8 girls; Male to Female ratio was 2.75: 1, which is similar to Hiradfar M et al [103] and slightly lesser than the literature reported ratio of 4:1 [8, 10] possibly because of the lesser number of patients. Of these patients 26 (86.7 %) were classic form of HD, 3 (10 %) long segment form and 1 (3.3 %) total colonic aganglionosis (TCA) which is similar to the study of Hiradfar M et al , where evaluated the 30 HD cases, there were 24 SHD cases (80%), 9 LHD cases (16.7%) and 1 TCA case (3.3%) [103]. The incidence (10%) of TCA is lower than that reported the Indian study by Kannaiyan L et al [105] which is again could be due to lesser number of patients.

Tissue samples of 30 cases were processed and evaluated for ganglion cells and nerve hypertrophy in all the four layers of bowel. However, some layers in full thickness biopsy specimens were not available for evaluation. The unavailable layers were excluded when the data was analysed for descriptive statistics. For example, muscularis propria was not included in biopsy of three patients.

Two features were evaluated in H&E, the absence of ganglion cells in submucosa and muscularis propria, and the presence of nerve bundle hypertrophy in these regions, for the diagnosis of HD. Though former is the pathognomonic feature for HD, the latter being a positive finding serves as a very useful additional feature for diagnosis. Of the 30 samples in our study, 5 cases showed ganglion cells in both the ganglionic and aganglionic segments, hence diagnosed as NHD. Of the remaining 25 samples of submucosa and 22 samples of muscularis propria, 19 in each layer showed nerve hypertrophy. Based on this the sensitivity, specificity and PPV for nerve bundle hypertrophy in diagnosing HD was 86.4%, 100% and 100% respectively (Table 6, 6A). The presence of hypertrophic nerve bundle was a strong positive factor for diagnosis while its absence did not accurately exclude HD.

Clinical short segment (SHD) disease showed absence of hypertrophic nerve bundles in around 15% of cases (Table 7) while the

extended segment disease (LHD/TCA), showed absence in 50%. This is similar to findings of Narayanan SK et al, who recorded lesser incidence (20%) of nerve bundle hypertrophy in the extended segment HD in a study of ninety two HD proved cases [71].

Calretinin IHC, similar to H&E, was also used to evaluate two features, viz., the immunoreactivity of ganglion cells in submucosa and muscularis propria, and immunoreactivity of intrinsic nerve fibres. When Calretinin staining was done, all the five cases with presence of ganglion cell in H&E also stained positive for Calretinin and showed immunoreactivity in nerve fibres as well, thus confirming the diagnosis of non-HD. Of 25 cases diagnosed as HD by ganglion cell negativity using IHC, three cases (12% cases) showed immunoreactivity in nerve fibres. However, the pattern of staining was found to be faint and punctate (Fig 21A&B) which was distinctly different from confluent, dark, granular immunostaining of normal nerve fibres. This showed that nerve fibres can show immunoreactivity falsely in cases of HD. Hiradfar et al [103] similarly showed false positive staining of nerve fibres in 2/ 30 cases (6.7%) of HD patients. Kapur et al [81] also recorded nerve fibre immunoreactivity in submucosal nerve fibres in the few of their HD patients. No explanation could be provided for this pattern by the authors. Guinard-Samuel et al [102] explained that this positive staining of some

nerve fibers could indicate the beginning of transitional zone. The nerve fibre immunoreactivity in HD had sensitivity and specificity of 88% and 100% respectively (Table 8).

H&E was compared with Calretinin IHC to find if IHC would fare better in diagnosing HD. Submucosa was available for evaluation on 30 samples and muscularis propria in 27 samples. Of these 57 observations, Observer 1 recorded 13 (23%) equivocal findings and observer 2 recorded 3(5%) equivocal findings (Table 9 and 10). There was no suspicious or equivocal finding using Calretinin IHC. Our findings showed a 100% and 90% sensitivity of H&E for Observer 1 and observer 2 respectively. However the sensitivity was poor.

Interobserver agreement was assessed between observer 1, a non experienced pathologist and observer 2, an experienced pathologist. The Cohen's kappa assessment showed a moderate agreement for H&E of 0.574(kappa) (95%CI 0.36-0.78) (Page no 71). However, observations of Calretinin IHC stained slides showed no discordance among the 30 cases. The kappa value of IHC showed a perfect agreement (Kappa=1, $p<0.05$) (Table 11 and 12). This is similar to the findings of Anbardar MH et al [109]. They showed interobserver agreement (Kappa) of 0.97 using Calretinin IHC in their study on 82 paraffin blocks.

Based on their observations, both the evaluators were required to give a final diagnosis of HD or non HD for all the cases. There was equivocal diagnosis in four cases by Observer 1 and for one case by observer 2 using H&E. Five cases were diagnosed as NHD and rest as HD by both observers. Calretinin confirmed all the five NHD diagnosis giving a specificity of 100%. Calretinin IHC confirmed all the suspicious diagnosis as HD. This showed that additional 13.3% (4 cases of 30) could be diagnosed when Calretinin IHC is used over H&E. This improved diagnosis is concordant with the study by Guinard et al, where of the 131 cases studied 12 additional cases which were equivocal by H&E could be diagnosed as HD with Calretinin IHC [103]. Kannaiyan et al in their study of 60 cases also showed all 15 cases with equivocal diagnosis by H&E could be diagnosed with accuracy with Calretinin IHC [105].

There are few limitations to our study. Our data shows Calretinin is a good adjunct to H&E in the diagnosis of HD. But this does not obviate the need for H&E and Calretinin IHC is still an additional investigation that would be required to improve the diagnosis. The specificity of H&E could not be calculated due to equivocal diagnosis in few cases. We could not get all the layers of bowel in all 30 patients, reducing the robustness of our descriptive statistics.

SUMMARY

- This retro-prospective study was done on full thickness rectal biopsy and resected specimens of patients with suspected Hirschsprung's disease (HD).

- The study was conducted at Coimbatore Medical College Hospital, Coimbatore and the study period being August 2016 – March 2018.

- The study was done to compare the diagnostic sensitivity of H&E and Calretinin IHC in suspected cases of HD and the staining pattern of ganglion cells in various layers of bowel tissue. Interobserver concordance in diagnosis was also evaluated between two pathologists for both H&E and IHC.

- Total of 30 cases of clinically suspected HD were taken for study. A sample from ganglionic and aganglionic segment were analyzed for each patient for the presence of ganglion cells and nerve bundle hypertrophy by H&E. Similarly, Calretinin was used to assess the ganglion cells and nerve fibre immunoreactivity in all the bowel layers.

- Both H&E and Calretinin assessment was done by two pathologists, one experienced (observer 2) and another in-training pathologist (observer 1), independently, blinded from each other.

- Descriptive statistics was used to assess the demographic parameters and diagnostic performance of Calretinin IHC. Cohen's kappa was used to calculate the interobserver agreement. Sensitivity and specificity analysis was done wherever appropriate.

- Most patients were neonates. 87% had short segment involvement. 73.3% and 26.7% specimens were full thickness biopsy and resection specimens respectively.

- Of the 30 specimens, 5 were diagnosed as Non-HD based on H&E and confirmed with Calretinin IHC. Of the 25 remaining patients, suspicious diagnosis was reported in four patients by Observer 1 and one patient by Observer 2. However, both pathologists could provide a definitive diagnosis of HD with Calretinin IHC in these cases. Hence, Calretinin IHC improved the sensitivity by 13.3% over H&E.

- There were around 13.8% equivocal observations by H&E for detecting ganglion cells, while no equivocal observations with Calretinin IHC.

- In H&E, nerve fibre hypertrophy as a predictor of HD showed a sensitivity and specificity of 86.4% and 100% respectively. Nerve immunoreactivity in IHC showed a sensitivity and specificity of 88% and 100% respectively.

- The Cohen's kappa assessment showed a moderate agreement for H&E of 0.574 (95%CI: 0.36-0.78), while IHC showed a perfect agreement of 1.

- Our findings suggest that Calretinin improves the sensitivity of diagnosing HD by 13% over H&E by providing confirmatory diagnosis for patients with equivocal findings.

- The agreement in diagnosing HD by an experienced and non-experienced pathologist also significantly improved ($p<0.05$) with the use of Calretinin IHC.(kappa = 0.574 for H&E and kappa=1 for IHC).

CONCLUSION

Our present study shows that Calretinin can be a useful adjunct to H&E in the diagnosis of HD, improving the diagnostic sensitivity (diagnose more cases), accuracy (confirm suspicious cases) and precision (reduce interobserver variation).

There was no interobserver variation between experienced and non-experienced pathologist in diagnosing HD using Calretinin IHC unlike H&E. So, Calretinin could prove to be a very useful adjunct marker in improving diagnostic accuracy in centres with lesser experience.

Calretinin IHC adds to the cost of diagnosis of HD. This additional cost could be justified by the improvement in sensitivity and accuracy which would save the patient from morbidity and expenses of a second look surgery.

There was 12% false positive immunoreactivity of nerve fibres to Calretinin IHC. Though the pattern of reactivity was different from that of non-HD cases, this warrants further study.

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MASTER CHART

S.no.	HPE no.	Age (Day)	Sex	Extent of HD	Specimen	Observer 1 (AP)																			
						H&E								Calretinin											
						Clinical aganglionic site				Clinical ganglionic site				Clinical aganglionic site						Clinical ganglionic site					
						Ganglion Cells		HT nerve fibre		Ganglion cells		HT nerve fibres		Ganglion Cells		Intrinsic nerve fibres				Ganglion cells		Intrinsic nerve fibres			
						SM	MP	SM	MP	SM	MP	SM	MP	SM	MP	LP	MM	SM	MP	SM	MP	LP	MM	SM	MP
1	2886/16	28	Male	SHD	resection	Pos	E	Pos	Neg	Pos	Pos	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
2	3105/16	7	Female	SHD	Biopsy	Neg	E	Neg	Pos	Pos	Pos	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
3	3354/16	30	Male	SHD	Biopsy	Neg	Neg	Pos	Pos	Neg	Pos	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
4	3774/16	7	Male	SHD	Biopsy	E	E	Neg	Pos	Pos	E	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
5	4033/16	15	Male	SHD	Biopsy	Neg	E	Pos	Pos	Pos	Pos	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Pos	Pos	Pos
6	0333/17	2	Female	SHD	Biopsy	E	Neg	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
7	0399/17	365	Male	SHD	resection	Pos	Pos	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
8	0445/17	154	Female	LHD	resection	Neg	Neg	Neg	Pos	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
9	0563/17	5	Male	SHD	Biopsy	Neg	Absent	Pos	Absent	Absent	E	Absent	Pos	Neg	Absent	Neg	Absent	Neg	Absent	Absent	Pos	Absent	Absent	Absent	Pos
10	0899/17	1460	Male	SHD	Biopsy	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
11	1237/17	3650	Female	SHD	resection	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Pos	Neg	Neg	Neg	Pos	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
12	1980/17	42	Male	SHD	Biopsy	E	Pos	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Pos	Absent	Absent	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
13	2215/17	2	Male	SHD	Biopsy	E	Neg	Neg	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
14	2216/17	3650	Female	SHD	Biopsy	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
15	2344/17	7	Male	SHD	Biopsy	E	Neg	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
16	2345/17	184	Female	SHD	resection	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
17	2469/17	7	Female	LHD	Biopsy	Neg	E	Neg	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
18	2487/17	4	Male	SHD	Biopsy	Pos	Pos	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
19	3239/17	215	Male	SHD	Biopsy	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
20	3300/17	3	Male	SHD	Biopsy	Neg	Neg	Neg	Neg	E	Absent	Neg	Absent	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Absent	Pos	Pos	Pos	Absent
21	3352/17	4	Male	SHD	Biopsy	Neg	Neg	Neg	Pos	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
22	3761/17	2	Male	SHD	Biopsy	E	Pos	Neg	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
23	3848/17	122	Male	SHD	resection	E	E	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Pos	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
24	3989/17	365	Male	SHD	Biopsy	Pos	Absent	Neg	Absent	Pos	Absent	Neg	Absent	Neg	Absent	Pos	Pos	Pos	Absent	Pos	Absent	Pos	Pos	Pos	Absent
25	4067/17	365	Male	SHD	resection	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
26	4385/17	6	Male	TCA	Biopsy	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos
27	0062/18	122	Female	SHD	Biopsy	Pos	Pos	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos
28	0192/18	365	Male	SHD	Biopsy	Neg	Absent	Pos	Absent	Pos	Pos	Neg	Neg	Neg	Absent	Neg	Neg	Neg	Absent	Pos	Pos	Pos	Pos	Pos	Pos
29	0212/18	60	Male	LHD	resection	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
30	0920/18	731	Male	SHD	Biopsy	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Pos	Pos	Pos

SHD= Short Segment Hirschprung disease
LHD= Long Segment Hirschprung disease
TCA= Total colonic Aganglionosis

E= equivocal
Absent= sample unavailable
Pos= positive
Neg= Negative

SM: Submucosa
MP: Muscularis propria
LP:Laminapropria
MM: muscularis mucosa

HT= Hypertrophic nerve bundles

S.no.	HPE no.	Age (Day)	Sex	Extent of HD	Specimen	Observer 2 (CL)																				Calretinin Immuno-reactivity Index
						H&E								Calretinin												
						Clinical aganglionic site				Clinical ganglionic site				Clinical aganglionic site						Clinical ganglionic site						
						Ganglion Cells		HT nerve fibre		Ganglion cells		HT nerve fibres		Ganglion Cells		Intrinsic nerve fibres				Ganglion cells		Intrinsic nerve fibres				
						SM	MP	SM	MP	SM	MP	SM	MP	SM	MP	LP	MM	SM	MP	SM	MP	LP	MM	SM	MP	
1	2886/16	28	Male	SHD	resection	E	Pos	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	3
2	3105/16	7	Female	SHD	Biopsy	Neg	Neg	Neg	Pos	Neg	E	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	3
3	3354/16	30	Male	SHD	Biopsy	Neg	Neg	Pos	Pos	E	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	3
4	3774/16	7	Male	SHD	Biopsy	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	3
5	4033/16	15	Male	SHD	Biopsy	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Pos	Pos	Pos	2
6	0333/17	2	Female	SHD	Biopsy	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	3
7	0399/17	365	Male	SHD	resection	Pos	Pos	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	2
8	0445/17	154	Female	LHD	resection	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	3
9	0563/17	5	Male	SHD	Biopsy	Neg	Absent	Neg	Absent	Absent	Neg	Absent	Neg	Neg	Absent	Neg	Neg	Neg	Absent	Absent	Neg	Absent	Absent	Absent	Neg	Neg
10	0899/17	1460	Male	SHD	Biopsy	Neg	Neg	Neg	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	3
11	1237/17	3650	Female	SHD	resection	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	3
12	1980/17	42	Male	SHD	Biopsy	Pos	Pos	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Pos	Absent	Absent	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	3
13	2215/17	2	Male	SHD	Biopsy	Neg	Neg	Pos	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	3
14	2216/17	3650	Female	SHD	Biopsy	E	Neg	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	3
15	2344/17	7	Male	SHD	Biopsy	Neg	Neg	Pos	Pos	E	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	2
16	2345/17	184	Female	SHD	resection	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	3
17	2469/17	7	Female	LHD	Biopsy	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	3
18	2487/17	4	Male	SHD	Biopsy	Pos	Pos	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	3
19	3239/17	215	Male	SHD	Biopsy	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	3
20	3300/17	3	Male	SHD	Biopsy	Neg	Neg	Pos	Neg	Neg	Absent	Neg	Absent	Neg	Neg	Pos	Neg	Pos	Neg	Pos	Absent	Pos	Pos	Pos	Absent	2
21	3352/17	4	Male	SHD	Biopsy	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
22	3761/17	2	Male	SHD	Biopsy	E	Neg	Neg	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	3
23	3848/17	122	Male	SHD	resection	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	3
24	3989/17	365	Male	SHD	Biopsy	Neg	Absent	Pos	Absent	Pos	Absent	Neg	Absent	Neg	Absent	Neg	Neg	Neg	Absent	Pos	Absent	Pos	Pos	Pos	Absent	3
25	4067/17	365	Male	SHD	resection	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
26	4385/17	6	Male	TCA	Biopsy	Neg	Neg	Pos	Pos	E	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	2
27	0062/18	122	Female	SHD	Biopsy	Pos	Pos	Neg	Neg	Pos	Pos	Neg	Neg	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	Pos	2
28	0192/18	365	Male	SHD	Biopsy	Neg	Absent	Pos	Absent	Pos	Pos	Neg	Neg	Neg	Absent	Neg	Neg	Neg	Absent	Pos	Pos	Pos	Pos	Pos	Pos	3
29	0212/18	60	Male	LHD	resection	Neg	Neg	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg
30	0920/18	731	Male	SHD	Biopsy	Neg	Neg	Pos	Pos	Pos	Pos	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Pos	Pos	Neg	Pos	Pos	Pos	2