Pediatric and Developmental Pathology
2020, Vol. 23(1) 8–22

DOI: 10.1177/1093526619892351
journals.sagepub.com/home/pdp
SAGE

Diagnosis of Hirschsprung Disease

Lusine Ambartsumyan[1], Caitlin Smith[2], and Raj P Kapur[3]

Abstract

Diagnosis or exclusion of Hirschsprung disease (HSCR) is a frequent exercise in any pediatric hospital. Although HSCR may present at different ages and with varied clinical findings, the most common presentation is a neonate with severe constipation or signs of intestinal obstruction. A variety of diagnostic tests including contrast enema and anorectal manometry may be used as diagnostic screens, but diagnosis ultimately rests upon histopathological evaluation of a rectal biopsy. For the experienced pathologist, conventional hematoxylin-and-eosin-stained sections often suffice to exclude HSCR or establish the diagnosis. However, ancillary diagnostic tests such as acetylcholinesterase histochemistry or calretinin immunohistochemistry are complementary and extremely helpful in some cases. In this Perspectives article, we review the clinical and pathological features of HSCR, highlight those that are found in most patients, and discuss how to address particularly challenging aspects of the diagnostic workup.

Keywords

Hirschsprung disease, acetylcholinesterase, rectal biopsy, calretinin, choline transporter, immunohistochemistry, diagnosis, anorectal manometry

Epidemiology and Clinical Presentations

Hirschsprung disease (HSCR) is a complex multigenetic disease characterized by the absence of intrinsic ganglion cells in the submucosal and myenteric plexuses of the intestinal tract. The aganglionic segment starts distally at the internal anal sphincter (IAS) and extends proximally to variable lengths of intestine. In 80% of affected children, the aganglionic segment is limited to the rectosigmoid (short-segment HSCR); in 3% to 10%, the entire colon is aganglionic (total colonic aganglionosis [TCA]); and in a tiny minority, aganglionosis extends to the proximal small intestine.[1–4] Very short-segment HSCR (vssHSCR) denotes an aganglionic distal rectal segment of $\leq$2 cm.[5] Mortality rates of 2% to 5% are reported despite advances in diagnostic, surgical, and medical care.[6,7] Children with HSCR have a significantly lower quality of life, with negative impacts on their social and emotional well-being and reduced physical activity.[8] Older age, fecal incontinence, and constipation have all been implicated in increased childhood social morbidity and parental anxiety and depression.[8–10]

The worldwide incidence of HSCR ranges from 1:5000 to 1:10 000 live births and varies among different ethnic groups (Northern European, 1.5:10 000; African American, 2.1:10 000; and Asian, 2.8:10 000).[2,11,12] A male-to-female ratio of 3:1 to 4:1 is observed largely in short-segment disease[1,2,12]; the sex bias diminishes in long-segment disease with a reported male-to-female ratio of 1:2 to 2:1.[1,3]

An increased index of suspicion for HSCR should be maintained for children with associated malformations. Nonsyndromic or isolated HSCR accounts for 70% of affected children, but approximately 30% of children affected with HSCR have an associated chromosomal (12%) and/or a congenital (18%) anomaly.[2,6,13] Trisomy 21 (Down syndrome, DS) is the most commonly associated chromosomal anomaly.[2,14] In a recent meta-analysis, Friedmacher and Puri[15] reported the incidence of DS in HSCR to be 7.32% and conversely the incidence of HSCR in DS to be 2.62%; hence, DS increases the risk

[1]Department of Gastroenterology, Seattle Children's Hospital and University of Washington, Seattle, Washington
[2]Department of Pediatric Surgery, Seattle Children's Hospital and University of Washington, Seattle, Washington
[3]Department of Pathology, Seattle Children's Hospital and University of Washington, Seattle, Washington

Corresponding Author:
Raj P Kapur, Seattle Children's Hospital, OC.8.720, 4800 Sand Point Way NE, Seattle, WA 98105, USA.
Email: raj.kapur@seattlechildrens.org

of HSCR by 50- to 100-fold.[6] Syndromes frequently associated with HSCR include Waardenburg-Shah syndrome, Goldberg-Shprintzen syndrome, multiple endocrine neoplasia 2A, congenital central hypoventilation syndrome, and Mowat-Wilson syndrome, among others.[2,6,11,14,16] Associated congenital anomalies may involve malformations of the cardiac, gastrointestinal, genitourinary, limb, and central nervous and peripheral neuronal systems.[2,11] Syndromic HSCR and nonsyndromic HSCR, as well as commonly associated anomalies in other organ systems, have been associated with pathogenic variants in multiple genes responsible for the development of the enteric nervous system.[2,17]

The genetics of HSCR are complex and involve multiple susceptibility loci. The summary genetic view of HSCR susceptibility is that it arises primarily from the segregation of alterations in multiple genes, both rare and common, most of which impair signal transduction by the RET receptor tyrosine kinase.[18,19] HSCR susceptibility in most nonsyndromic cases and a subset of syndromic patients is explained by the combinatorial effects of common *low-penetrance* noncoding variants at one or more of the following gene loci: *RET, NRG1*, and *SEMA3*. In contrast, major chromosomal alterations (eg, trisomy 21) or rare *high-penetrance* mutations in 12 genes (*RET, GDNF, NRTN, SOX10, EDNRB, EDN3, ECE1, ZFHX1B, PHOX2B, KBP, TCF4*, and *L1CAM*) are often observed in syndromic HSCR.[2,18,20] These complex genetics explain the varied recurrence rates and incomplete penetrance observed in families and may contribute to differences in clinical outcome.

Clinical Presentation

Although HSCR has been historically considered to be a disease of the neonatal period, a retrospective review of national inpatient databases reported that only 6.5% of patients present within the first week of life with mean and median ages of presentation to be 3 years and 1 year, respectively.[21] Approximately 40% present by 6 months of age, 50% by 1 year of age, and 60%, 80%, and 93% by ages 2 years, 7 years, and 13 years, respectively. Clinical symptoms vary by age at presentation and extent of the aganglionic segment. Neonates commonly present with delayed passage of meconium (>48 h), feeding intolerance, abdominal distention, and bilious emesis, symptoms that are concerning for intestinal obstruction.[6,22] Bowel perforation of cecum, ascending colon, or appendix may be the presenting symptom in up to 5% of neonates with HSCR. Children with longer segment of aganglionosis, such as TCA, commonly present as neonates with symptoms of intestinal obstruction[22]; however, delayed presentation of greater than 6 months has been reported in up to 14%.[23]

Beyond the neonatal period, infants and children may present with long-standing mild-to-severe constipation that is refractory to oral laxatives, dependent on rectal therapies, and associated with vomiting, abdominal distention, and growth failure.[6,22,24] Their course may be complicated by acute intestinal obstruction, recurrent fecal impactions, and acute life-threatening or chronic enterocolitis. Hirschsprung-associated enterocolitis (HAEC) is characterized by explosive diarrhea (often bloody), fever, and abdominal distention. HAEC presents preoperatively in 6% to 60% of patients and postoperatively in 25% to 37% with reported mortality rates of 1% to 10%.[25–29]

HSCR beyond childhood and notably in adulthood is considered rare.[30–32] In their systematic review and meta-analysis of HSCR in adults, Doodnath and Puri[31] reported that disease was limited to the rectum and rectosigmoid in 79.8% and 12.5% of patients respectively. Most had symptoms during the neonatal period followed by lifelong constipation, abdominal distention, and pain that was poorly responsive to laxatives or enemas. Therefore, HSCR should be suspected in any adolescent or adult with symptoms of intractable constipation that began in childhood.

Diagnostic Procedures

Patients with high index of suspicion for HSCR should undergo a methodical diagnostic evaluation. Two screening tests available for the diagnostic workup of HSCR are contrast enema (CE) and anorectal manometry (ARM). Patients who have findings concerning for HSCR are recommended to undergo a rectal suction biopsy (RSB). If the results of the RSB are equivocal, then a full-thickness biopsy (FTB) is recommended to establish the diagnosis. For older patients (eg, > 1 year of age), some centers advocate FTB over RSB at the outset.

Contrast Enema

A CE is a widely available screening test for HSCR. Like ARM, it is less invasive than a rectal biopsy but does require exposure to radiation. Unlike ARM, it is widely available in most centers but does require an experienced radiologist to perform and accurately interpret the study. During a CE, water-soluble contrast is instilled into the colon using a catheter that is placed inside the anus followed by live fluoroscopic imaging.

Findings that are suggestive of HSCR include the presence of a radiographical transition zone (TZ) with proximal dilated bowel, microcolon, retention of contrast on postevacuation film, irregular colonic contractions, mucosal irregularity, and an abnormal rectosigmoid ratio.[33–36] The rectosigmoid ratio, which is calculated as the diameter of the rectum divided by the sigmoid colon during CE, is normally greater than 1; a value less than 1 suggests HSCR. The radiographic location of the TZ has been shown to correlate with the length of the aganglionic

segment, specifically of the rectosigmoid segment, and may aid in surgical procedure planning.[34,37] However, correlation was lower in segments proximal to the rectosigmoid and in children younger than 3 months of age. Muller et al.[38] reported a global agreement of 58.1% (κ=0.39) between the TZ and length of aganglionosis in their cohort of 79 patients with HSCR. This further highlights the importance of intraoperative biopsies to direct surgical planning. In patients with long-segment HSCR and specifically in those with TCA, the TZ may be falsely or inaccurately reported, thus resulting in diagnostic and treatment delay.[39,40] Also, in children with vssHSCR, a TZ can be inapparent by CE. Furthermore, use of an enema, irrigation, or a rectal examination to decompress the distal colon prior to a CE and the inflation of a balloon on the catheter used to instill the contrast can distort the TZ in patients with vssHSCR and lead to inaccurate results.[35,41]

The reported diagnostic accuracy of CE in the literature is variable.[33,36,37,42–46] In a systematic review of the literature, de Lorijn et al.[41] reported that CE had lower mean sensitivity and specificity of 70% and 83%, respectively, when compared to ARM and RSB. Furthermore, CE in the neonatal period has been shown to be less reliable than in older children.[42,44–46] Age less than 30 days was shown to be a predictor of a false-positive result,[33] and CE results in those less than 30 days of age was reported to have a 7-fold higher probability of false-negative results.[37] The diagnostic accuracy of the rectosigmoid ratio and of the TZ in the diagnosis of HSCR was reported to be lower in the neonatal period and statistically lower in infancy when compared to older children.[44] Ultimately, in a patient with concern for HSCR, a normal CE does not rule out HSCR, and a rectal biopsy is recommended.

Anorectal Manometry

ARM is used to evaluate the voluntary and the involuntary properties of the anorectum.[47–49] It is the most common motility test performed in children and requires little preparation. It is less invasive than a rectal biopsy, and unlike CE, it does not require radiation exposure. Performance and interpretation of ARM require operator skill and knowledge, expertise offered only in select gastrointestinal motility referral centers. Patient cooperation is essential for a successful and accurate test, but sedation or anesthesia can be used for children who may not cooperate during the study. The choice of sedation is crucial as it may affect the rectoanal inhibitory reflex (RAIR) or lower the intraanal pressures and result in difficulty assessing for the RAIR.

During ARM, a flexible catheter, with a nonlatex balloon at its distal end, is introduced into the rectum. Sensors spaced at predetermined intervals along the shaft of the catheter allow for continuous measurement of intraanal pressures during the study (Figure 1(A)). The manometric assessment of the anorectal function includes measurement of the length of the anal canal and resting pressures, RAIR, rectal sensation, and last, the ability to squeeze and simulate defecation.[48,49] The RAIR is the reflex relaxation of the IAS in response to rectal distention. This reflex is present in individuals with normal intrinsic innervation of the intestine and is absent in those with HSCR (Figure 1(B)).[50,51]

The North American Society for Pediatric Gastroenterology, Hepatology and Nutrition recommends ARM as the initial screening test for HSCR in centers with manometric capability.[52] The minimum age when ARM can be performed varies between centers. In our center, we perform screening ARM for HSCR in

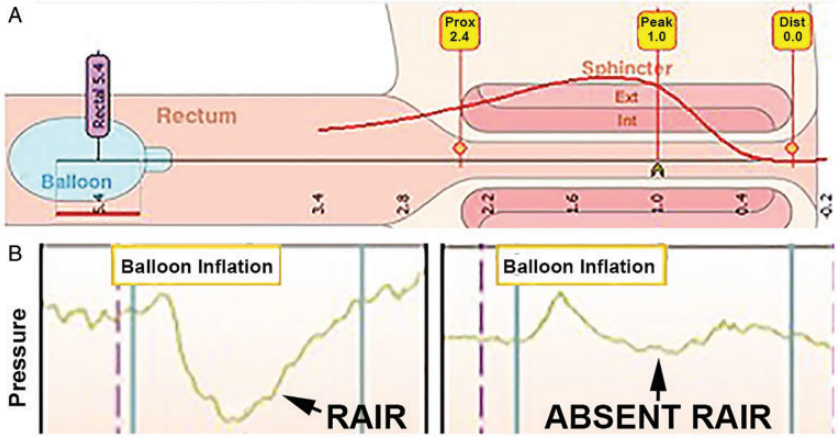

Figure 1. Anorectal manometry. A, Diagram from anorectal manometry showing location of balloon in rectum and sensors (yellow squares) in anal canal (numbers are distances from anal verge in cm). B, Representative single sensor pressure tracings from a different individual with a normal RAIR (left) and a patient with an absent RAIR (right).
RAIR, rectoanal inhibitory reflex.

infants who are older than 3 months of age. If the results of ARM are consistent with HSCR or are equivocal, then the patient is referred for rectal biopsy. In a systematic review, de Lorijn et al.[41] compared the diagnostic accuracy of ARM, CE, and RSB in infants suspected of HSCR. They reported that the RSB was the most accurate test with the highest mean sensitivity of 93% and mean specificity of 98%. The diagnostic accuracy of ARM was only slightly less: mean sensitivity and specificity of 91% and 94%, respectively. However, the reported diagnostic accuracy of ARM versus RSB varies considerably, and ARM has been reported to have a significantly lower rate of specificity and positive predictive value in some studies.[53,54] Additionally, the accuracy of ARM in the diagnosis of HSCR in neonates conflicts in the literature.[55–57] In a subgroup analysis of diagnostic accuracy of ARM in infants younger than 6 months, de Lorijn et al.[41] found overall lower sensitivity of 88% and specificity of 89% but no significant difference from the total group. Similarly, Meinds et al.[54] did not demonstrate a significant difference between the 3 age groups in their cohort.

False-negative results of ARM have been attributed to displacement of the catheter or relaxation of the external anal sphincter (EAS).[41,52] False-positive results have been attributed to air leak, catheter and equipment problems, and insufficient inflation of the balloon.[41] High-resolution ARM introduced in 2008 has addressed the limitations associated with conventional catheters.[58–60] There are greater number of sensors placed at close intervals circumferentially and along the longitudinal axis that improve the resolution of the study, thereby making the study easier to perform and more efficient. ARM is considered the screening test of choice for HSCR, and a positive test should be confirmed with RSB.

Rectal Suction Biopsy

In 1948, Whitehouse and Kernohan[61] published the first detailed case series to firmly establish aganglionosis of the distal rectum and a variable length of contiguous bowel as the primary phenotypic feature of HSCR. Since then the definitive diagnosis or exclusion of HSCR has depended on microscopic evaluation of a rectal biopsy. Initially, incisional biopsies were performed under anesthesia with direct visualization of the anal canal and distal rectum.[62] Incisional biopsies yielded generous samples of tissue and usually were deep enough to encompass both smooth muscle layers of the muscularis propria and intervening myenteric plexus. Later, suction rectal biopsies were introduced as a diagnostic method that could be performed without general anesthesia and with less potential for significant bleeding or infection.[63] Although incisional or forceps biopsies are sometimes advocated, especially for older patients, suction rectal biopsy has become the diagnostic mainstay for most patients.[64]

There has been some evidence that the age of the infant influences the success of obtaining an adequate or diagnostic tissue sample on suction rectal biopsy. Meinds et al. performed a retrospective review of 441 patients who underwent a suction rectal biopsy from 1975 to 2011. The average sensitivity of suction rectal biopsy in patients aged less than 39 days was only 50%, in contrast to 88% sensitivity in older patients.[65] Diagnostic specificity was high (~95%) independent of age. This has not been our experience or the published experience of other groups, who report much higher diagnostic sensitivity even in very young neonates.[66–68] Other published data indicate varied levels of diagnostic accuracy but generally favor a trend toward increasing inconclusive results in older infants.[69]

At our institution, suction rectal biopsy is the initial and primary modality for obtaining tissue to rule out HSCR in most patients younger than 6 months of age. The procedure can be performed in the clinic without anesthesia in a relatively rapid fashion. Our practice for patients older than 12 months is to perform a full-thickness rectal biopsy in the operating room. Patients aged 6 to 12 months may be less cooperative with a clinic procedure without sedation and should be evaluated on a case-by-case basis.

The suction rectal biopsy technique was first described by Dobbins and Bill[63] in 1965. The technique requires a specialized rectal biopsy instrument (Figure 2), which is shaped like a narrow-barreled handgun with a hole in the side of the barrel. Different models exist, but inherent in most is some type of disposable, razor-edged ''cut-and-capture'' cartridge that attaches to the end of the barrel. The outside of barrel or attachment is marked at centimeter intervals, and a syringe can be connected to the lumen of the barrel to create the vacuum needed to draw rectal mucosa and submucosa through the side port and into the lumen. After application of lubricant, the barrel of the instrument is inserted into the anal canal. The cartridge markings are key as they mark the level past the anal verge from which the biopsy will be taken. The first biopsy is taken at the level with the instrument inserted 2 cm (2 markings on outside of the barrel) passed the anal verge into the anal canal. If this is performed correctly, the first biopsy will be obtained approximately 1 cm proximal to the dentate line. Suction is applied to instrument with a 50- to 60-mL syringe, and the instrument is triggered to slice across the side port. While the mucosa and submucosa are suctioned into the barrel, the edge of the cartridge slices and collects a small amount of mucosa and submucosa as specimen. This is repeated 3 cm and 4 cm internal to the anal verge (estimated 2 cm and 3 cm proximal to dentate line) on subsequent biopsies. During each biopsy, the side port on the barrel of the

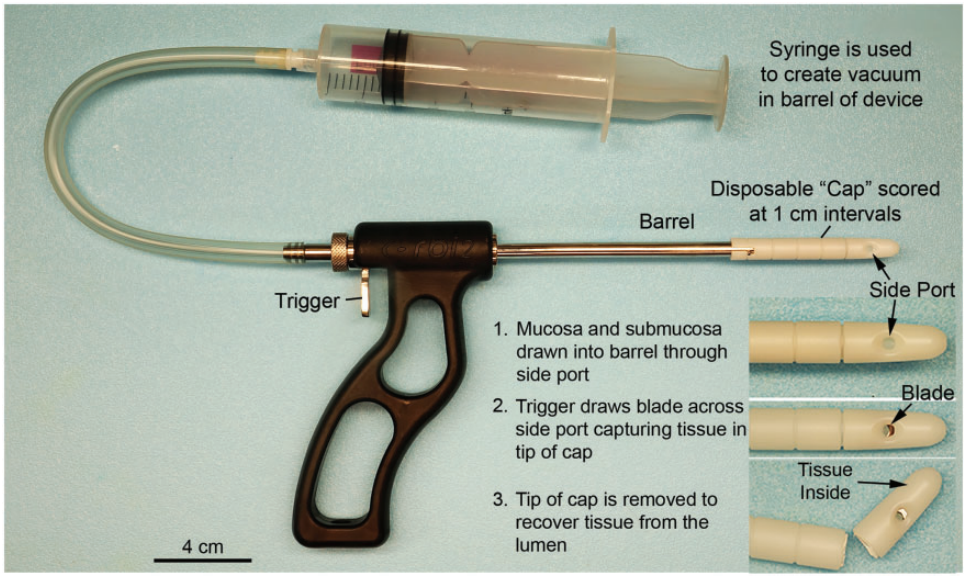

Figure 2. Suction rectal biopsy device.

device should be gently pressed against the posterior rectum to ensure sufficient tissue is obtained. Once adequate tissue is obtained at the 3 levels (2 cm, 3 cm, and 4 cm above the anal verge), they are placed in formalin and sent to pathology for analysis. It is important to submit these biopsies in separate containers so that the findings can be correlated with each specified location. If acetylcholinesterase histochemistry will be performed, an additional biopsy should be obtained (typically at 3 cm) and sent to the laboratory on a moist gauze pad to be frozen immediately in appropriate medium.

An open rectal biopsy is performed under general anesthesia in the operating room. The patient is placed into the lithotomy position. A Lone Star® retractor (Cooper Surgical Medical Devices, Trumbull, CT) is placed to expose the anal canal. The pins of the retractor are situated so as to identify and preserve the dentate line. The proximal and distal margins of the rectal biopsy specimen are marked with sutures, and additional traction suture is placed in the center of the specimen site. The specimen is taken sharply with scissors and sutured closed for hemostasis. The biopsy site will bleed, and care and preparation for closing the biopsy site should be taken prior to obtaining the specimen to ensure blood loss is minimal. Bleeding and perforation are known complications for both suction and open rectal biopsies, although both in relatively low frequency.[69,70]

Diagnostic Pathology

Rectal Biopsy Pathology

Surgical pathology approaches to the diagnosis of HSCR have evolved in parallel with the biopsy techniques. Early descriptions focused primarily on aganglionosis and other alterations in the myenteric plexus, whereas contemporary diagnostic techniques rely entirely on alterations in the submucosa and mucosa. Some diagnostically useful features are only resolved with enzyme histochemistry or immunohistochemistry (IHC), and no one standardized approach is universally employed. Fortunately, reliable diagnosis can be achieved for the majority of patients by various alternative methods. The most critical variable is the pathologist's experience and familiarity with the strengths and limitations of the approach used in their clinical and laboratory setting. In this *Perspectives* article, we describe the most commonly applied practice, which is based primarily on formalin-fixed paraffin-embedded (FFPE) tissue samples ± a frozen sample for acetylcholinesterase enzyme (AChE) histochemistry.

Normal Anorectal Anatomy

In order to perform and interpret rectal biopsies, it is very important to know normal anorectal anatomy. Unfortunately, the subject is complicated by inconsistent use of definitions and nomenclature for landmarks in the anorectal canal. Claus Fenger[71] has written an excellent review of this subject, and his conclusions are the basis for the anatomical terminology employed here (Figure 3). Like more proximal large intestine, the distal rectum is lined internally by colonic mucosa and surrounded by muscularis propria with an inner circular layer (muscularis interna) and outer longitudinal layer (muscularis externa). The IAS is a circular "collar" formed by the thick inferior end of the muscularis interna. This sphincter surrounds and defines the length of the *anal canal*, which connects the distal rectum to perianal skin.

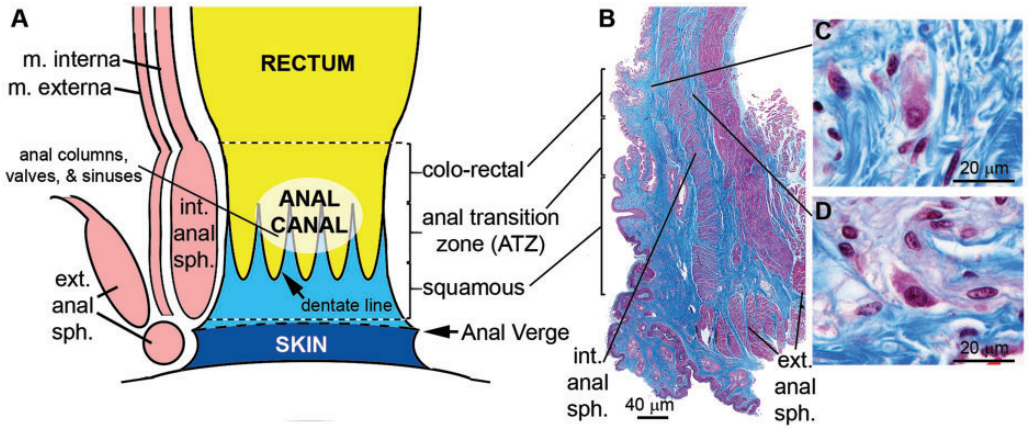

Figure 3. A, Diagrammatic representation of anorectal anatomy to illustrate key landmarks including the anal canal, which extends the length of the internal anal sphincter and is lined superiorly by colorectal mucosa and inferiorly by squamous mucosa, with anal transition mucosa in between. B, Longitudinal histologic section (trichrome stain) through the anal canal highlights the inferior-most submucosal (C) and myenteric (D) ganglion cells that are located deep to colorectal mucosa, superior to the anal columns and anal transition mucosa. ext., external; int., internal; m., muscularis; sph., sphincter.

Superiorly, the anal canal is lined by colorectal mucosa, whereas the inferior end of the canal is squamous mucosa. In between is the *anal transitional zone* (ATZ), where a mixture of 2 more epithelial types (colonic, squamous, transitional, or other variants) is found. Within the ATZ, longitudinal columns are separated inferiorly by valves and sinuses, and the base of these valves form the *dentate (pectinate) line*. At the inferior end of the anal canal, an indentation (intersphincteric groove, Hilton's white line, mucocutaneous junction) may be present at junction of the squamous mucosa and skin, where the IAS abuts the distalmost aspect of the EAS. The *anal verge* is an ill-defined skin-covered region, immediately below the intersphincteric groove.

Multiple studies have shown that the density of ganglion cells declines toward the inferior end of the normal rectum.[72–77] These observations led to the concept of an inferior rectal zone of physiologic hypoganglionosis or possibly aganglionosis and the recommendation that biopsies to exclude HSCR should be taken proximal to this zone. Aldridge and Campbell suggested at least 2 cm "above the anal valves [dentate line]";[72] others specified > 2 cm above the mucocutaneous junction/anal verge.[63,76] Thoughtful examination of the basis for these recommendations is important because biopsy taken superior to a very short pathogenic aganglionic segment may lead to a false-negative diagnosis.

One of the earliest detailed studies used to define the physiologic hypoganglionic zone was by Aldridge and Campbell,[72] who examined cadaveric anorectal specimens from 20 patients, who ranged from premature infants to 15 years of age. For each of the specimens, 9 longitudinal sections through the distal rectum and surgical anal canal and 9 transverse hemicircumferential sections (0.5, 1.5, and 2.5 cm proximal to the dentate line, 3 sections at each location) were examined microscopically. The sections were 5 μm thick and stained with hematoxylin-and-eosin (H&E). Although a gap of up to 17 mm was found between the base of the anal valves (dentate line) and the inferior-most submucosal ganglion cell in the 3 longitudinal sections from some specimens, ganglion cells were present <5 mm from the dentate in some of the corresponding transverse sections. For 2 specimens, H&E-stained serial sections were performed to quantify ganglion cells in 4 mm-long × 10 mm-thick patches of superficial submucosa. Again, superficial ganglion cells were sparse, but present, in the terminal 0.5 cm above the dentate line. On the basis of these and other observations, Aldridge and Campbell concluded as follows:

- The terminal 0.5 cm above the valves (dentate line, not anal verge) should be considered "hypoganglionic" rather than aganglionic.
- The hypoganglionic zone is lined by transitional ("intermediate") epithelium in some specimens.
- Ganglion cells in the myenteric plexus generally extend further caudally beyond submucosal ganglion cells, so "if ganglion cells are found in the submucosal plexus there will also be ganglion cells in the myenteric plexus."

Subsequent studies of cadavers have confirmed that the distal-most rectum is hypoganglionic relative to more proximal colon but focused on FTB or deep forceps biopsy, which include abundant submucosa and the myenteric plexus.[73–77] In most of these investigations, the investigators focused on myenteric ganglia, samples with transitional mucosa were not explicitly excluded, and/or samples were not sectioned exhaustively in a manner similar to how suction rectal biopsies are handled in many laboratories. Hence, it is unclear whether a

suction biopsy with adequate submucosa and only rectal (not transitional) mucosa can be "too low" and give a false impression of aganglionosis, despite exhaustive histological evaluation. Perhaps the nearest approximation to a suction rectal biopsy study of non-HSCR controls was published by Venugopal et al.,[67] who analyzed cadaveric samples taken with laryngeal biopsy forceps (deeper and larger than suction biopsy) at 0.5 to 1 cm and 1 to 1.5 cm superior to the pectinate. Using no more than 25 H&E-stained sections and excluding biopsies with anal TZ mucosa, they identified submucosal ganglion cells in 68/68 (100%) of the specimens with submucosal tissue.

As discussed above, most surgeons are taught to biopsy at least 2 cm above the anal verge. Choice of landmark is critical because the position of the dentate line is estimated but not visible when suction biopsy is performed, and, depending on the age of the patient, the dentate line may be located 1 to 9 mm superior to the anal verge (mucocutaneous junction). In a neonate, a biopsy 2 cm proximal to the dentate line is likely to be in rectal mucosa and may be in the TZ of a patient with vssHSCR, whereas a biopsy 2 cm superior to the anal verge is likely to be close to the top of the anal canal and may be in the anal TZ. Furthermore, the interface between transitional epithelium and colorectal mucosa is irregular, and the length of the different zones in the canal varies greatly between individuals. One reason it is advisable to obtain suction biopsies at multiple levels (eg, 2-, 3-, and 4-cm proximal to the anal verge) is to reduce the likelihood that all biopsies will be either too low (transitional mucosa) or too high.[78]

Suction Rectal Biopsy

Confident diagnosis or exclusion of HSCR with a suction rectal biopsy depends on the following factors:

(a) Identification of an unequivocal ganglion cell (Figure 4): Recognition of a ganglion cell in an H&E-stained section is not difficult for the experienced observer, particularly if the histological quality of the tissue section is good. Classic cytological features include abundant cytoplasm, an eccentric round nucleus, conspicuous nucleolus, perinuclear pallor, and peripheral chromatin. Usually, but not always, ganglion cells are grouped in ganglia and/or in contact with neuropil (neurites and glia). Cytologically immature ganglion cells with less cytoplasm and stippled nuclear chromatin and indistinct nucleolus can pose a challenge and may predominate in the very young or premature infant. However, with practice, even immature ganglion cells can be identified with confidence. In rare instances, it may be helpful to use IHC, possibly even on a destained H&E-section that contains an equivocal cell, to be definitive. Robust neuron-specific markers include Hu C/D (cell soma) and Phox2b (nuclear). If a ganglion cell is identified, HSCR is usually excluded, unless features of TZ are identified, in which case vssHSCR must be considered (see below and Kapur et al.—fourth paper in this issue). In rare instances, cytomegalovirus (CMV) infection can produce cytological changes in nonneuronal cells that closely mimic the appearance of mature ganglion cells (Figure 5(A)). Usually a large nuclear or

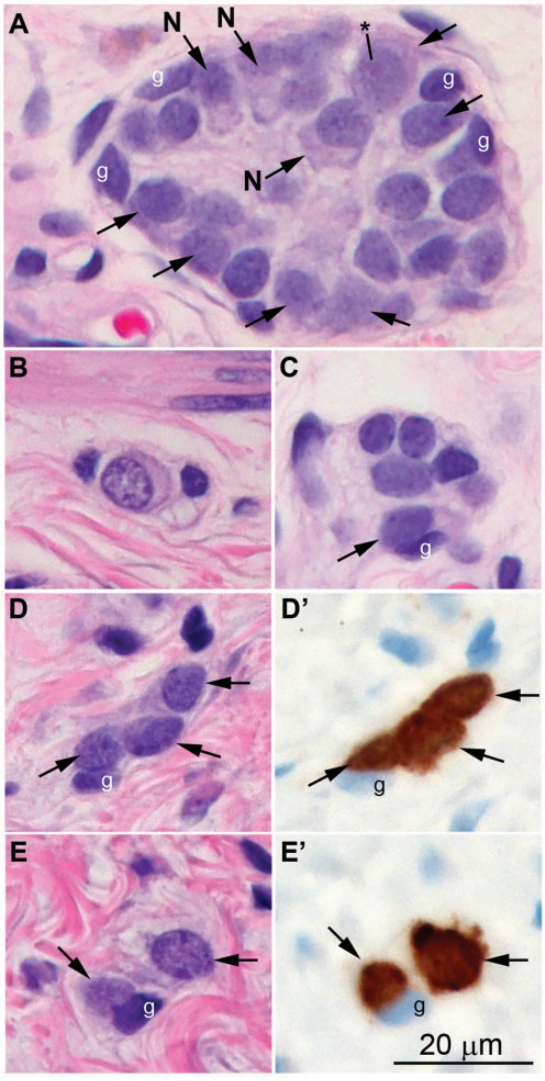

Figure 4. Ganglion cell cytology. A to E, Representative H&E appearances of submucosal ganglia illustrating varying degrees of cytological immaturity. Many examples of definitive neurons (arrows) are shown in which the nuclei are round and chromatin is finely granular. Some of these ganglion cells (N) have visible cytoplasm with eccentric nuclei and Nissl substance ± conspicuous nucleoli (asterisks in A). In contrast, enteric glia cells (g) have more condensed chromatin, oval nuclei, and no easily identified cytoplasmic boundaries. The ganglia shown in D and E were destained and immunolabeled with anti-Hu, a neuronal cell body-specific marker, which highlights the neurons (D' and E'). All images at same magnification.

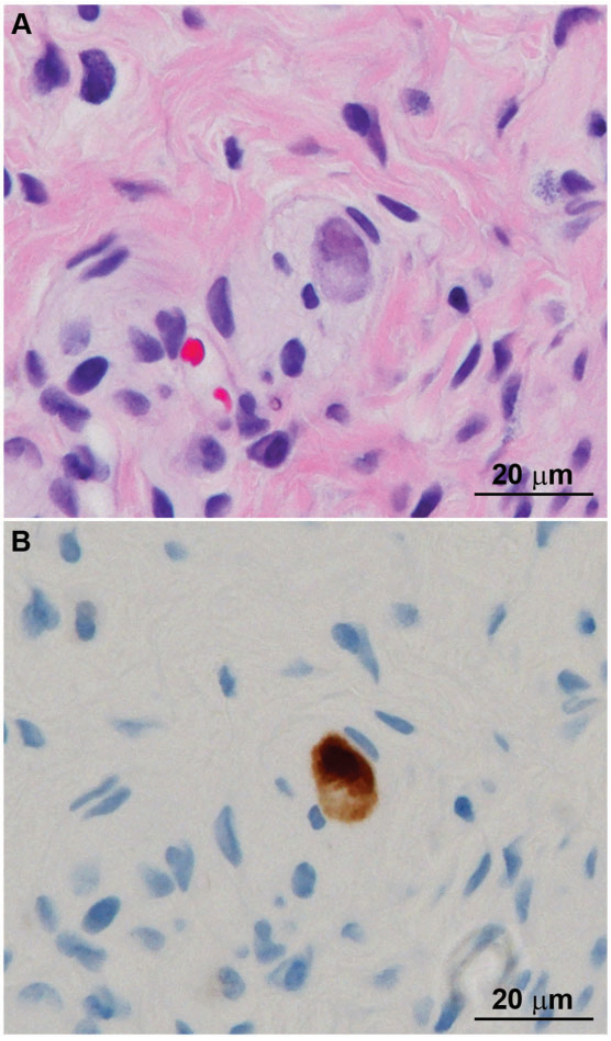

Figure 5. Cytomegaloviral cytopathology mimicking a mature ganglion cell in the aganglionic biopsy of a patient with HSCR. A, H&E appearance of atypical cell in a submucosal nerve. B, Same field as in A after section was destained and immunolabeled with a cytomegalovirus-specific antibody.

small, eosinophilic, cytoplasmic viral inclusions are present, which distinguish CMV cytopathology from neuronal differentiation. If a biopsy has other features to suggest HSRC and CMV is a consideration, IHC, either for a neuron-specific antigen (eg, Hu C/D, Phox2B) or CMV-specific antigen (Figure 5(B)), can resolve the issue. More often a careful search of all available tissue sections will identify unequivocal CMV cytopathology and allow for 2 diagnoses, HSCR and active CMV infection.

(b) Appropriate mucosa (location): The surface of the biopsy should be lined entirely by colonic mucosa. Biopsies from the portion of the normal anorectal canal that is lined by transitional or squamous mucosa may be aganglionic and therefore are regarded as ''inadequate'' unless a ganglion cell is identified.

(c) Adequate submucosa: Confident exclusion of ganglion cells generally requires a suction biopsy 2 to 3 mm in greatest dimension and 50% or more submucosa. Histological sampling of an adequate biopsy is begun by routinely cutting 50 to 75 H&E-stained sections (4 to 5 μm thick).[78] The overwhelming majority of sections should contain some submucosa, and one-third or more of the cut surface should be submucosa in the majority of sections. However, when relying only on H&E sections, it is often prudent to cut additional levels or exhaust the block. If no ganglion cell is identified and overt submucosal nerve hypertrophy is present, the diagnosis can be established with no need for further evaluation. Unfortunately, some biopsies lack these unequivocal diagnostic findings or have atypical features that require additional study.

(d) Submucosal nerve hypertrophy: In the aganglionic rectal submucosa from a patient with HSCR, many large nerves are usually present (Figure 6). These nerves probably arise from nonintestinal autonomic ganglia, primarily in the pelvis. They retain some of the ultrastructural and immunohistochemical properties of peripheral nonenteric nerves including perineurium, which expresses the glucose transporter, Glut1 (Figure 6).[79–81] Submucosal nerve hypertrophy affects large and small nerves such that the submucosa appears crowded with nerves of various large diameters, some significantly larger than normally encountered in distal rectum. In a neonate, it is unusual to encounter a submucosal nerve with a diameter >40 μm, except in the aganglionic rectosigmoid colon of patients with HSCR.[82] Nerve caliber increases with age, and it is common to encounter nerves >40 μm, including some with Glut1-positive perineuria, in the rectum of a toddler or older individual.[83,84] Significant nerve hypertrophy is a reliable diagnostic finding in most patients but is paradoxically absent in some patients with TCA.[85–87]

(e) Application of ancillary techniques (Table 1): H&E-based diagnosis/exclusion of HSCR by an experienced pathologist is fairly reliable and may be justified, particularly if adequate submucosa and diagnostic findings are present in numerous sections from each of multiple suction biopsies. However, when sections from a biopsy sample only a small amount of submucosa or an aganglionic biopsy does not contain large submucosal nerves, it is often comforting and sometimes necessary to apply (immune)histochemistry to facilitate a diagnosis. In practice, most centers that use one or both of these ancillary methods do so routinely on all suction biopsies, in part to maintain experience with the method and interpretation. The most widely applied ancillary methods are AChE histochemistry and calretinin IHC.

AChE histochemistry highlights abnormally coarse and dense cholinergic mucosal innervation, which probably represents the mucosal projections from

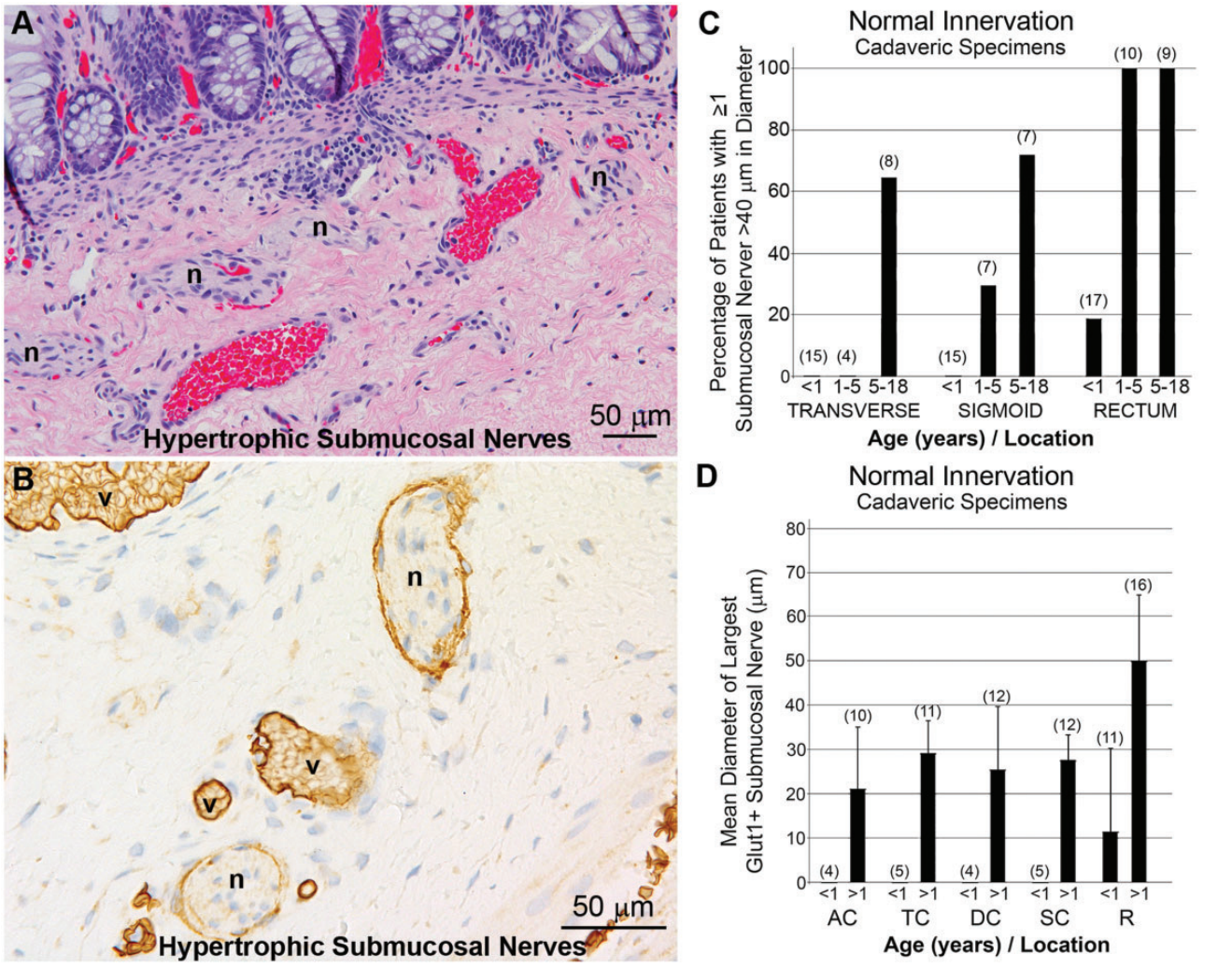

Figure 6. A, Hypertrophic submucosal nerves (n) are almost always present in the aganglionic rectum of patients with HSCR. B, These large nerves (n) are circumscribed by Glut1-immunoreactive perineuria and are easily distinguished from Glut1-positive erythrocytes in adjacent blood vessels (v). C, Normative data collected from cadaveric controls show that rectal nerves >40 µm in diameter are rarely present in the rectums of infants under the age of 1 year but are common in rectums of older patients. D, Similarly, Glut1-positive nerves, when present in the distal rectum, are fewer (not show) and smaller in infants (error bars are standard deviations). Numbers of patients are shown in parentheses. The graph shown in D is a portion of Figure 1 from Kapur et al.[83] with permission.
AC, ascending colon; DC, descending colon; R, rectum; SC, sigmoid colon; TC, transverse colon.

Table 1. Ancillary Diagnostic Approaches to Hirschsprung Disease.

Acetylcholinesterase Enzyme Histochemistry	Calretinin Immunohistochemistry	Choline Transporter Immunohistochemistry
Frozen sections	Formalin-fixed paraffin sections	Formalin-fixed paraffin sections
Unique reagents and methodology	Routine in most laboratories	Routine in most laboratories
Manual processing	Can be done with automated immunostainer	Can be done with automated immunostainer
Abnormal innervation equates with gain of histochemical staining	Abnormal innervation equates with loss of immunoreactivity	Abnormal innervation equates with gain of immunoreactivity
Usually easy to interpret but requires regular practice	Usually easy to interpret without much practice	Often requires considerable practice to interpret correctly

hypertrophic submucosal nerves (Figure 7).[88] AChE histochemistry is performed on frozen sections, typically with unfixed tissue, and is not compatible with traditional formalin fixation and paraffin embedding. The histochemical procedure has no other application in the laboratory so that maintenance of technical and interpretive proficiency can be a problem. In a skilled laboratory with an experienced pathologist, AChE histochemistry is a sensitive and specific diagnostic approach, but invariably some equivocal staining patterns are encountered, and most laboratories do not rely on AChE histochemistry alone.[89]

In most instances, AChE histochemistry is performed on a separate biopsy (as opposed to a divided biopsy) from those used for H&E analysis. Occasionally, this leads to discrepant findings, especially if a patient has vssHSCR and biopsies are close to the TZ. Particular attention should be given to submucosal nerve hypertrophy, which usually extends into the distal TZ, where large nerves coexist with ganglion cells and abnormal cholinergic mucosal innervation may be present.[90] It is prudent to examine H&E-stained frozen sections from the tissue submitted for AChE histochemistry and to submit residual frozen tissue for formalin fixation and routine processing. Choline transporter (ChT) IHC (see below) may help to confirm abnormal cholinergic innervation in sections from FFPE biopsies. If discrepancies persist, they may need to be resolved by additional suction or incisional biopsies.

Calretinin IHC labels a subset of enteric neurons including their cell bodies and neurites. In normal ganglionic bowel, calretinin-immunoreactive nerves are readily identified in the muscularis mucosae and lamina propria, although they may be sparse near the anorectal transitional zone. Complete absence of calretinin-positive mucosal nerves is indicative of aganglionic rectal tissue, although nerves in the submucosa may contain

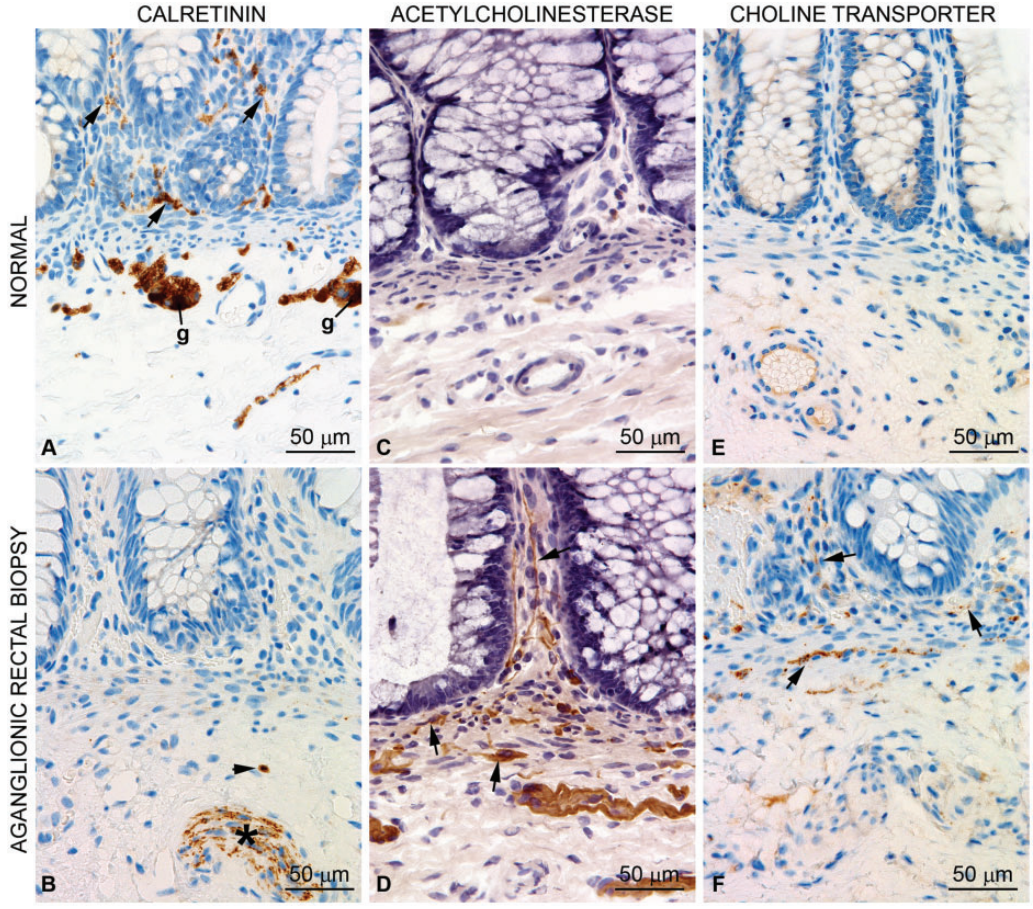

Figure 7. Ancillary diagnostic techniques. A, Calretinin immunohistochemistry highlights mucosal nerves (arrows) and a subset of superficial submucosal ganglion cells (g) in normal rectal tissue. B, Immunoreactive mucosal nerves and ganglion cells are completely absent in a biopsy from aganglionic rectum, although expression is present in enlarged submucosal nerves (asterisk) and mast cells (arrowhead). Acetylcholinesterase histochemistry (C and D) or choline transporter immunohistochemistry (E and F) demonstrate sparse or absent cholinergic mucosal nerves in biopsies from normal ganglionic rectum (C and E), as opposed to numerous coarse mucosal nerves (arrows in D and F) in aganglionic rectum.

immunoreactive fibers (Figure 7).[91,92] Mast cells also express calretinin but are round or ovoid with fine cytoplasmic staining that should not be confused with the linear profiles of dense granular staining in mucosal nerves. Mast cells provide an internal control for calretinin IHC, but undue reliance on their staining should be avoided since limited data exist as to whether calretinin in neurons may be more vulnerable to artefactual loss (eg, degradation) than mast cell calretinin. Calretinin IHC appears to be as diagnostically specific and sensitive as AChE histochemistry[91,93] but can be conducted with FFPE tissue using methodology available in most pathology laboratories. False-negative results (retained calretinin-immunoreactive mucosal innervation) occur rarely and usually result either from enteric neurons that project inferiorly into the aganglionic segment in vssHSCR or a distal skip area.[5,94] False-positive results (loss of calretinin immunoreactivity) can result when tissue is frozen and thawed,[95] so calretinin IHC should not be applied to biopsies after they are used for AChE histochemistry.

A potential alternative to AChE is ChT IHC.[90] Like AChE, ChT is expressed in the abnormal cholinergic nerves found in aganglionic rectal mucosa. In contrast with AChE histochemistry, ChT IHC can be performed with FFPE tissue sections. In our experience, ChT IHC may be more difficult to interpret than AChE histochemistry but with regular practice has comparable diagnostic value. Like AChE histochemistry, ChT IHC demonstrates larger and more numerous nerves in the mucosa of aganglionic bowel (Figure 7), a finding that is particularly useful with biopsies from patients with vssHSCR, because calretinin may be misleading in this context.

Incisional Rectal Biopsy

Incisional rectal biopsies are either longitudinal strips of superficial and deep submucosa biopsy or FTB that include muscularis propria and myenteric plexus. In contrast with suction biopsies, these require examination under anesthesia and are done with direct visualization of the dentate line, so the location is precise. Incisional biopsies are favored in older patients (>1 year) because with patient growth suction biopsies yield a smaller proportion of the submucosa[96] and can be helpful to confirm or exclude vssHSCR, when suction rectal biopsy findings are equivocal. Incisional biopsy is also part of the evaluation of some post-pullthrough patients who continue to have serious obstructive symptoms.[25,97]

Incisional biopsies are much larger and deeper than suction rectal biopsies, so fewer H&E-stained sections (eg, 2 slides with 2 ribbons per slide) are generally sufficient. Incisional biopsies are typically large enough to bisect perpendicular to the mucosal surface. A portion may be frozen for AChE histochemistry, or both halves fixed and embedded in paraffin. Orientation of the tissue to retain the relationships between mucosa, submucosa, and muscularis propria/myenteric plexus is important, and use of a hand lens or dissection microscope is helpful when handling these specimens to ensure the tissue is divided and oriented well. Often biopsies thought by the surgeon to be full-thickness include only submucosa and mucosa but are still adequate for HSCR diagnosis. In other respects, the diagnostic findings and use of calretinin IHC and/or AChE histochemistry for HSCR are identical to suction biopsies.

Rectal Biopsy Report

Surgical pathology reports from rectal biopsies should, at a minimum, include the location and type of each biopsy, whether or not a ganglion cell or submucosal nerve hypertrophy was identified, and the results of any ancillary staining. Examples of suggested formats for the diagnostic lines in a report are as follows:

Rectum, "2 cm from anal verge," suction biopsy:

- Ganglion cells present
- No submucosal nerve hypertrophy
- Normal pattern of calretinin-immunoreactive mucosal innervation

Rectum, "2 cm from anal verge," suction biopsy: findings consistent with HSCR

- No ganglion cell identified
- Abundant hypertrophic submucosal nerves
- Absent calretinin-immunoreactive mucosal innervation

How Does a Pathologist Know if Aganglionic Biopsy Is From the Physiologic Hypoganglionic Zone?

In the authors' experience, when suction biopsies are taken at estimated distances of 2, 3, and 4 cm above the anal verge and exhaustively sectioned as needed, at least one submucosal ganglion cell will almost always be identified in each biopsy, provided only colonic mucosa is present and the biopsy has adequate submucosa. The exception of course is a patient with vssHSCR, in which the 2-cm ± more proximal biopsies will be aganglionic, but nerve hypertrophy and abnormal cholinergic mucosal innervation are present. Therefore, physiologic hypoganglionosis as an explanation for inability to identify a ganglion cell in a low biopsy with only colorectal mucosa is generally not a significant concern unless (a) the biopsy contains a marginal amount of submucosa, (b) exhaustive sections with submucosa have not been obtained, (c) the patient is older (eg, > 1 year) and the hypoganglionic zone and interganglionic spacing are proportionately longer, and/or (d) neither the aganglionic

Table 2. Rectal Biopsies With Equivocal or Discordant Diagnostic Features of HSCR.

Finding	Diagnostic Consideration(s)	Possible Resolution
Only equivocal (indefinite) ganglion cells in H&E-stained sections	- Immature ganglion cells, especially in a premature infant, are more difficult to distinguish from inflammatory or endothelial cells - CMV cytopathy can mimic ganglion cell cytology (Figure 5)	- Exhaust block with additional sections - Destain section(s) with equivocal cells and perform IHC with neuron-specific antibody (eg, anti-Phox2b and anti-Hu) - Presence of unequivocal submucosal nerve hypertrophy favors HSCR - Rebiopsy patient
One or more biopsy with adequate submucosa and no ganglion cells but intact calretinin-positive mucosal innervation	- Patient may have vssHSCR, and calretinin-positive mucosal nerves descend from the transition zone (more superior biopsy(ies) may have ganglion cells) - Patient may have a distal skip area (source of calretinin-positive mucosal nerves in adjacent aganglionic rectum)	- Exhaust block with additional sections - Presence of unequivocal submucosal nerve hypertrophy favors HSCR - AChE histochemistry (if frozen tissue available) or ChT IHC; positive result favors HSCR - Incisional or full-thickness biopsy to exclude vssHSCR - Anorectal manometry (absent RAIR favors HSCR)
Unequivocal submucosal nerve hypertrophy (AChE histochemistry and ChT IHC usually abnormal), but ganglion cells and calretinin-positive mucosal nerves are present	- Patient may have vssHSCR, and biopsy is from the transition zone (most inferior biopsy(ies) may lack ganglion cells) - Biopsy is from a distal skip area	- AChE histochemistry (if frozen tissue available) or ChT IHC; positive result favors HSCR - Incisional or full-thickness biopsy to exclude vssHSCR - Anorectal manometry (absent RAIR favors HSCR) - For older patient, threshold for submucosal nerve hypertrophy should be higher
Suboptimal submucosal tissue, no identifiable ganglion cell, indefinite submucosal nerve hypertrophy, absent calretinin-immunoreactive mucosal innervation	- Most likely HSCR (truly absent calretinin-immunoreactive mucosal innervation is highly specific for aganglionic bowel)	- Verify staining IHC-positive control section (ideally ganglionic gut on same slide) - Look for calretinin IHC-positive mast cells and submucosal nerves as internal control - Repeat calretinin IHC - AChE histochemistry (if frozen tissue available) or ChT IHC; positive result favors HSCR - Low threshold to request additional biopsies, especially if only one biopsy was obtained - Anorectal manometry (absent RAIR favors HSCR)

Abbreviations: AChE, acetylcholinesterase enzyme; ChT, choline transporter; CMV, cytomegalovirus; H&E, hematoxylin-and-eosin; HSCR, Hirschsprung disease; IHC, immunohistochemistry; RAIR, rectoanal inhibitory reflex; vssHSCR, short-segment HSCR.

biopsy nor ganglionic biopsies immediately upstream show submucosal nerve hypertrophy or abnormal cholinergic mucosal innervation. In any of the latter scenarios, it is best to have a low threshold to rebiopsy and to consider incisional biopsy, particularly in an older patient.

Common Challenging Scenarios

Diagnosis or exclusion of HSCR based on rectal biopsy is usually straightforward. Classic pathology findings include absent ganglion cells, abundant large submucosal nerves, absent calretinin-immunoreactive mucosal innervation, and abnormally abundant, coarse cholinergic mucosal nerves, as demonstrated by AChE histochemistry or ChT IHC. However, equivocal or discordant findings, which may represent unusual variations of HSCR or related conditions, occur in a significant minority of cases. These ''difficult'' cases may benefit from additional studies as indicated in Table 2.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) received no financial support for the research, authorship, and/or publication of this article.

References

1. Badner JA, Sieber WK, Garver KL, Chakravarti A. A genetic study of Hirschsprung disease. *Am J Hum Genet*. 1990;46:568–580.
2. Amiel J, Sproat-Emison E, Garcia-Barcelo M, et al. Hirschsprung disease, associated syndromes and genetics: a review. *J Med Genet*. 2008;45:1–14.
3. Laughlin DM, Friedmacher F, Puri P. Total colonic aganglionosis: a systematic review and meta-analysis of long-term clinical outcome. *Pediatr Surg Int*. 2012;28:773–779.

4. Urla C, Lieber J, Obermayr F, et al. Surgical treatment of children with total colonic aganglionosis: functional and metabolic long-term outcome. *BMC Surg*. 2018;18:58.
5. Kapur RP. Calretinin-immunoreactive mucosal innervation in very short-segment Hirschsprung disease: a potentially misleading observation. *Pediatr Dev Pathol*. 2014;17:28–35.
6. Heuckeroth RO. Hirschsprung disease – integrating basic science and clinical medicine to improve outcomes. *Nature Rev Gastroenterol Hepatol*. 2018;15:152–167.
7. Granstrom AL, Svenningsson A, Nordenskjold A, Wester T. Population-based study shows that Hirschsprung disease does not have a negative impact on education and income. *Acta Paediatrica*. 2016;105:1508–1512.
8. Collins L, Collis B, Trajanovska M, et al. Quality of life outcomes in children with Hirschsprung disease. *J Pediatr Surg*. 2017;52:2006–2010.
9. Sood MR, Cocjin J, Di Lorenzo C, Narasimha Reddy S, Flores AF, Hyman PE. Ileal manometry in children following ileostomies and pull-through operations. *Neurogastroenterol Motil*. 2002;14:643–646.
10. Kyrklund K, Neuvonen MI, Pakarinen MP, Rintala RJ. Social morbidity in relation to bowel functional outcomes and quality of life in anorectal malformations and Hirschsprung's disease. *Eur J Pediatr Surg*. 2018;28:522–528.
11. Parisi MA, Kapur RP. Hirschsprung disease overview. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, ed. *GeneReviews at GeneTests-GeneClinics: Medical Genetics Information Resource* [database online]. Seattle, WA: University of Washington; 1997–2002. http://www.genetests.org. Accessed November 18, 2019.
12. Bradnock TJ, Knight M, Kenny S, Nair M, Walker GM. Hirschsprung's disease in the UK and Ireland: incidence and anomalies. *Arch Dis Child*. 2017;102:722–727.
13. Moore SW, Zaahl M. Clinical and genetic differences in total colonic aganglionosis in Hirschsprung's disease. *J Pediatr Surg*. 2009;44:1899–1903.
14. Burkardt DD, Graham JM Jr, Short SS, Frykman PK. Advances in Hirschsprung disease genetics and treatment strategies: an update for the primary care pediatrician. *Clin Pediatr*. 2014;53:71–81.
15. Friedmacher F, Puri P. Hirschsprung's disease associated with Down syndrome: a meta-analysis of incidence, functional outcomes and mortality. *Pediatr Surg Int*. 2013;29:937–946.
16. Ke J, Zhu Y, Miao X. The advances of genetics research on Hirschsprung's disease. *Pediatr Invest*. 2018;2:189–195.
17. Sergi CM, Caluseriu O, McColl H, Eisenstat DD. Hirschsprung's disease: clinical dysmorphology, genes, micro-RNAs, and future perspectives. *Pediatr Res*. 2017;81:177–191.
18. Chakravarti A, McCallion AS, Lyonnet S. *Hirschsprung disease*. In: Valle D, et al., ed. *The Online Metabolic & Molecular Bases of Inherited Disease*. New York, NY: McGraw-Hill; 2014. doi:10.1036/ommbid.291.
19. Tilghman JM, Ling AY, Turner TN, et al. Molecular genetic anatomy and risk profile of Hirschsprung's disease. *N Engl J Med*. 2019;380:1421–1432.
20. Alves MM, Sribudiani Y, Brouwer RW, et al. Contribution of rare and common variants determine complex diseases-Hirschsprung disease as a model. *Dev Biol*. 2013;382:320–329.
21. Aboagye J, Goldstein SD, Salazar JH, et al. Age at presentation of common pediatric surgical conditions: reexamining dogma. *J Pediatr Surg*. 2014;49:995–999.
22. Langer JC. Hirschsprung disease. *Curr Opin Pediatr*. 2013;25:368–374.
23. Moore SW. Total colonic aganglionosis and Hirschsprung's disease: shades of the same or different? *Pediatr Surg Int*. 2009;25:659–666.
24. Nurko S. Motility disorders in children. *Pediatr Clin North Am*. 2017;64:593–612.
25. Langer JC, Rollins MD, Levitt M, et al. Guidelines for the management of postoperative obstructive symptoms in children with Hirschsprung disease. *Pediatr Surg Int*. 2017;33:523–526.
26. Gosain A, Frykman PK, Cowles RA, et al. Guidelines for the diagnosis and management of Hirschsprung-associated enterocolitis. *Pediatr Surg Int*. 2017;33:517–521.
27. Jiao CL, Chen XY, Feng JX. Novel insights into the pathogenesis of Hirschsprung's-associated enterocolitis. *Chin Med J*. 2016;129:1491–1497.
28. Demehri FR, Halaweish IF, Coran AG, Teitelbaum DH. Hirschsprung-associated enterocolitis: pathogenesis, treatment and prevention. *Pediatr Surg Int*. 2013;29:873–881.
29. Gosain A. Established and emerging concepts in Hirschsprung's-associated enterocolitis. *Pediatr Surg Int*. 2016;32:313–320.
30. Martinez JP. Adult Hirschsprung's disease. *Can J Emerg Med Care*. 2015;17:704–705.
31. Doodnath R, Puri P. A systematic review and meta-analysis of Hirschsprung's disease presenting after childhood. *Pediatr Surg Int*. 2010;26:1107–1110.
32. Grover K, Ahlawat SK. Hirschsprung disease in adults. *South Med J*. 2009;102:127–128.
33. Diamond IR, Casadiego G, Traubici J, Langer JC, Wales PW. The contrast enema for Hirschsprung disease: predictors of a false-positive result. *J Pediatr Surg*. 2007;42:792–795.
34. Chen X, Xiaojuan W, Zhang H, et al. Diagnostic value of the preoperatively detected radiological transition zone in Hirschsprung's disease. *Pediatr Surg Int*. 2017;33:581–586.
35. Carroll AG, Kavanagh RG, Ni Leidhin C, Cullinan NM, Lavelle LP, Malone DE. Comparative effectiveness of imaging modalities for the diagnosis of intestinal obstruction in neonates and infants: a critically appraised topic. *Acad Radiol*. 2016;23:559–568.
36. Peyvasteh M, Askarpour S, Ostadian N, Moghimi MR, Javaherizadeh H. Diagnostic accuracy of barium enema findings in Hirschsprung's disease. *Arq Brasil Cir Digest*. 2016;29:155–158.
37. Frongia G, Gunther P, Schenk JP, et al. Contrast enema for Hirschsprung disease investigation: diagnostic accuracy and validity for subsequent diagnostic and surgical planning. *Eur J Pediatr Surg*. 2016;26:207–214.
38. Muller CO, Mignot C, Belarbi N, Berrebi D, Bonnard A. Does the radiographic transition zone correlate with the level of aganglionosis on the specimen in Hirschsprung's disease? *Pediatr Surg Int*. 2012;28:597–601.
39. Chen JZ, Jamieson DH, Skarsgard ED. Does pre-biopsy contrast enema delay the diagnosis of long segment Hirschsprung's disease? *Eur J Pediatr Surg*. 2010;20:375–378.

40. Moore SW. Total colonic aganglionosis and Hirschsprung's disease: a review. *Pediatr Surg Int*. 2015;31:1–9.
41. de Lorijn F, Kremer LC, Reitsma JB, Benninga MA. Diagnostic tests in Hirschsprung disease: a systematic review. *J Pediatr Gastroenterol Nutr*. 2006;42:496–505.
42. De Lorijn F, Reitsma JB, Voskuijl WP, et al. Diagnosis of Hirschsprung's disease: a prospective, comparative accuracy study of common tests. *J Pediatr*. 2005;146:787–792.
43. Reid JR, Buonomo C, Moreira C, Kozakevich H, Nurko SJ. The barium enema in constipation: comparison with rectal manometry and biopsy to exclude Hirschsprung's disease after the neonatal period. *Pediatr Radiol*. 2000;30:681–684.
44. Garcia R, Arcement C, Hormaza L, et al. Use of the recto-sigmoid index to diagnose Hirschsprung's disease. *Clin Pediatr*. 2007;46:59–63.
45. Stranzinger E, DiPietro MA, Teitelbaum DH, Strouse PJ. Imaging of total colonic Hirschsprung disease. *Pediatr Radiol*. 2008;38:1162–1170.
46. Putnam LR, John SD, Greenfield SA, et al. The utility of the contrast enema in neonates with suspected Hirschsprung disease. *J Pediatr Surg*. 2015;50:963–966.
47. Camilleri M, Bharucha AE, di Lorenzo C, et al. American Neurogastroenterology and Motility Society consensus statement on intraluminal measurement of gastrointestinal and colonic motility in clinical practice. *Neurogastroenterol Motil*. 2008;20:1269–1282.
48. Di Lorenzo C, Hillemeier C, Hyman P, et al. Manometry studies in children: minimum standards for procedures. *Neurogastroenterol Motil*. 2002;14:411–420.
49. Rodriguez L, Sood M, Di Lorenzo C, Saps M. An ANMS-NASPGHAN consensus document on anorectal and colonic manometry in children. *Neurogastroenterol Motil*. 2017;29. doi:10.1111/nmo.12944.
50. Siddiqui A, Rosen R, Nurko S. Anorectal manometry may identify children with spinal cord lesions. *J Pediatr Gastroenterol Nutr*. 2011;53:507–511.
51. Morera C, Nurko S. Rectal manometry in patients with isolated sacral agenesis. *J Pediatr Gastroenterol Nutr*. 2003;37:47–52.
52. Tabbers MM, DiLorenzo C, Berger MY, et al. Evaluation and treatment of functional constipation in infants and children: evidence-based recommendations from ESPGHAN and NASPGHAN. *J Pediatr Gastroenterol Nutr*. 2014;58:258–274.
53. Jarvi K, Koivusalo A, Rintala RJ, Pakarinen MP. Anorectal manometry with reference to operative rectal biopsy for the diagnosis/exclusion of Hirschprung's disease in children under 1 year of age. *Int J Colorectal Dis*. 2009;24:451–454.
54. Meinds RJ, Trzpis M, Broens PMA. Anorectal manometry may reduce the number of rectal suction biopsy procedures needed to diagnose hirschsprung disease. *J Pediatr Gastroenterol Nutr*. 2018;67:322–327.
55. Iwai N, Yanagihara J, Tokiwa K, Deguchi E, Perdzynski W, Takahashi T. Reliability of anorectal manometry in the diagnosis of Hirschsprung's disease. *Z Kinderchir*. 1988;43:405–407.
56. Emir H, Akman M, Sarimurat N, Kilic N, Erdogan E, Soylet Y. Anorectal manometry during the neonatal period: its specificity in the diagnosis of Hirschsprung's disease. *Eur J Pediatr Surg*. 1999;9:101–103.
57. Huang Y, Zheng S, Xiao X. Preliminary evaluation of anorectal manometry in diagnosing Hirschsprung's disease in neonates. *Pediatr Surg Int*. 2009;25:41–45.
58. Lee TH, Bharucha AE. How to perform and interpret a high-resolution anorectal manometry test. *J Neurogastroenterol Motil*. 2016;22:46–59.
59. Lee YY, Erdogan A, Rao SS. High resolution and high definition anorectal manometry and pressure topography: diagnostic advance or a new kid on the block? *Curr Gastroenterol Rep*. 2013;15:360.
60. Basilisco G, Bharucha AE. High-resolution anorectal manometry: an expensive hobby or worth every penny? *Neurogastroenterol Motil*. 2017;29. doi:10.1111/nmo.13125.
61. Whitehouse FR, Kernohan JW. Myenteric plexus in congenital megacolon. *Arch Intern Med*. 1948;82:75–11.
62. Swenson O, Fisher JH, Macmahon HE. Rectal biopsy as an aid in the diagnosis of Hirschsprung's disease. *N Engl J Med*. 1955;253:632–635.
63. Dobbins WO, Bill AH. Diagnosis of Hirschsprung's disease excluded by suction rectal biopsy. *N Engl J Med*. 1965;272: 990–996.
64. Muise ED, Cowles RA. Rectal biopsy for Hirschsprung's disease: a review of techniques, pathology, and complications. *World J Pediatr*. 2016;12:135–141.
65. Meinds RJ, Kuiper GA, Parry K, et al. Infant's age influences the accuracy of rectal suction biopsies for diagnosing of Hirschsprung's disease. *Clin Gastroenterol Hepatol*. 2015;13: 1801–1807.
66. Noblett HR. A rectal suction biopsy tube for use in the diagnosis of Hirschsprung's disease. *J Pediatr Surg*. 1969;4: 406–409.
67. Venugopal S, Mancer K, Shandling B. The validity of rectal biopsy in relation to morphology and distribution of ganglion cells. *J Pediatr Surg*. 1981;16:433–437.
68. Alexandrescu S, Rosenberg H, Tatevian N. Role of calretinin immunohistochemical stain in evaluation of Hirschsprung disease: an institutional experience. *Int J Clin Exp Pathol*. 2013;6: 2955–2961.
69. Muise ED, Hardee S, Morotti RA, Cowles RA. A comparison of suction and full-thickness rectal biopsy in children. *J Surg Res*. 2016;201:149–155.
70. Brady AC, Saito JM, Lukas K, et al. Suction rectal biopsy yields adequate tissue in children. *J Pediatr Surg*. 2016;51: 966–969.
71. Fenger C. Histology of the anal canal. *Am J Surg Pathol*. 1988; 12:41–55.
72. Aldridge RT, Campbell PE. Ganglion cell distribution in the normal rectum and anal canal: a basis for the diagnosis of Hirschsprung's disease by anorectal biopsy. *J Pediatr Surg*. 1968;3:475–489.
73. Tafazzoli K, Soost K, Wessel L, Wedel T. Topographic peculiarities of the submucous plexus in the human anorectum – consequences for histopathologic evaluation of rectal biopsies. *Eur J Pediatr Surg*. 2005;15:159–163.
74. Hofmann S, Orestano F. Histology of the myenteric plexus in relation to rectal biopsy in congenital megacolon. *J Pediatr Surg*. 1967;2:575–577.
75. Ricciardi R, Counihan TC, Banner BF, Sweeney WB. What is the normal aganglionic segment of anorectum in adults? *Dis Colon Rectum*. 1999;42:380–382.
76. Weinberg AG. The anorectal myenteric plexus: its relation to hypoganglionosis of the colon. *Am J Clin Pathol*. 1970;54: 637–642.

77. Bodian M, Stephens FD, Ward BCH. Hirschsprung's disease and idiopathic megacolon. *Lancet*. 1949;1:6–11.
78. Qualman SJ, Jaffe R, Bove KE, Monforte-Munoz H. Diagnosis of Hirschsprung disease using the rectal biopsy: multi-institutional survey. *Pediatr Dev Pathol*. 1999;2:588–596.
79. Baumgarten HG, Holstein AF, Stelzener F. Nervous elements in the human colon of Hirschsprung's disease. *Virchows Arch (Pathol Anat)*. 1973;358:113–136.
80. Wedel T, Holschneider AM, Krammer H-J. Ultrastructural features of nerve fascicles and basal lamina abnormalities in Hirschsprung disease. *Eur J Pediatr Surg*. 1999;9:75–82.
81. Kakita Y, Oshiro K, O'Briain DS, Puri P. Selective demonstration of mural nerves in ganglionic and aganglionic colon by immunohistochemistry for glucose transporter-1: prominent extrinsic nerve pattern staining in Hirschsprung disease. *Arch Pathol Lab Med*. 2000;124:1314–1319.
82. Monforte-Munoz H, Gonzales-Gomez I, Rowland JM, Landing BH. Increased submucosal nerve trunk caliber in aganglionosis. A ''positive'' and objective finding in suction biopsies and segmental resections in Hirschsprung's disease. *Arch Pathol Lab Med*. 1998;122:721–725.
83. Kapur RP, Arnold MA, Conces MR, et al. Remodeling of rectal innervation after pullthrough surgery for Hirschsprung disease: relevance to criteria for the determination of retained transition zone. *Pediatr Dev Pathol*. 2019;22:292–303.
84. Kapur RP. Submucosal nerve diameter of greater than 40 um is not a valid diagnostic index of transition zone pull-through. *J Pediatr Surg*. 2016;51:1585–1591.
85. Narayanan SK, Soundappan SS, Kwan E, Cohen RC, Charlton A, Cass DT. Aganglionosis with the absence of hypertrophied nerve fibres predicts disease proximal to rectosigmoid colon. *Pediatr Surg Int*. 2016;32:221–226.
86. Meier-Ruge W, Hunziger O, Tobler H-J, Walliser C. The pathophysiology of aganglionosis of the entire colon (Zuelzer-Wilson syndrome): morphometric investigations of the extent of sacral parasympathetic innervation of the circular muscles of the aganglionic colon. *Beitr Path Bd*. 1972;147:228–236.
87. Matsuda H, Hirato J, Kuroiwa M, Nakazato Y. Histopathological and immunohistochemical study of the enteric innervations among various types of aganglionoses including isolated and syndromic Hirschsprung disease. *Neuropathology*. 2006;26:8–23.
88. Pacheco MC, Bove KE. Variability of acetylcholinesterase hyperinnervation patterns in distal rectal suction biopsy specimens in Hirschsprung disease. *Pediatr Dev Pathol*. 2008;11:274–282.
89. Agrawal RK, Kakkar N, Vasishta RK, Kumari V, Samujh R, Rao KL. Acetylcholinesterase histochemistry (AChE) – a helpful technique in the diagnosis and in aiding the operative procedures of Hirschsprung disease. *Diagn Pathol*. 2015;10:208.
90. Kapur RP, Raess PW, Hwang S, Winter C. Choline transporter immunohistochemistry: an effective substitute for acetylcholinesterase histochemistry to diagnose Hirschsprung disease with formalin-fixed paraffin-embedded rectal biopsies. *Pediatr Dev Pathol*. 2017;20:308–320.
91. Kapur RP, Reed RC, Finn LS, Patterson K, Johanson J, Rutledge JC. Calretinin immunohistochemistry versus acetylcholinesterase histochemistry in the evaluation of suction rectal biopsies for Hirschsprung disease. *Pediatr Dev Pathol*. 2009;12:6–15.
92. Guinard-Samuel V, Bonnard A, De Lagausie P, et al. Calretinin immunohistochemistry: a simple and efficient tool to diagnose Hirschsprung disease. *Mod Pathol*. 2009;22:1379–1384.
93. Holland SK, Ramalingam P, Podolsky RH, Reid-Nicholson MD, Lee JR. Calretinin immunostaining as an adjunct in the diagnosis of Hirschsprung disease. *Ann Diagn Pathol* 2011;15:323–328.
94. Coe A, Avansino JR, Kapur RP. Distal rectal skip-segment Hirschsprung disease and the potential for false-negative diagnosis. *Pediatr Dev Pathol*. 2016;19:123–131.
95. Lim KH, Wan WK, Lim TKH, Loh AHL, Nah SA, Chang KTE. Primary diagnosis of Hirschsprung disease – calretinin immunohistochemistry in rectal suction biopsies, with emphasis on diagnostic pitfalls. *World J Pathol*. 2014;3:14–22.
96. Vadva Z, Nurko S, Hehn R, Vargas SO. Rectal suction biopsy in patients with previous anorectal surgery for Hirschsprung disease. *J Pediatr Gastroenterol Nutr*. 2017;65:173–178.
97. Chumpitazi BP, Nurko S. Defecation disorders in children after surgery for Hirschsprung disease. *J Pediatr Gastroenterol Nutr*. 2011;53:75–79.